U N I V E R S I T Y of E D I N B U R G H

The contribution of plant cells and their enzymes to the biochemical changes which take place in the ensilage process.

by

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CONTENTS

INTRODUCTION

The majority of investigations of the ensilage process have been concerned with the effect of various conditions eg. temperature, moisture, aeration, and composition of the crop, on the subsequent chemical or bacteriological changes in the silo. In some cases both these aspects have been studied simultaneously. The interpretation of the data obtained in such work has been hampered by the difficulty of deciding whether any particular chemical change was the result of the activity of plant cells or of micro-organisms. The main object of the work described in this thesis was to remove this difficulty.

Since the metabolisms of both plant cells and micro-organisms are subject to destruction or inhibition by heat, antiseptics and the like, any attempt to differentiate between the activities of these two agents by changing the environment is unlikely to yield conclusive results. Nevertheless, a number of investigators have attacked the problem along these lines. Their work is discussed in the first section of this introduction. The second section deals with those investigations in which an attempt is made to correlate chemical changes with bacteriological changes in normal silage. Other approaches which have been made to the problem are dealt/

dealt with in the third section. In section four a review of relevant studies in plant physiology is presented.

1. Investigations involving the addition of biostatic substances to the fodder before ensiling.

The possible role of plant cells in the ensilage process could not be appreciated until the anaerobic respiration of plant tissue had been established by the work of Lechartier & Bellamy (1869) and Pasteur (1872). Before this information became available it was postulated that the acidification of silage was the result of bacterial activity and that the aerobic respiration of the plant cells was responsible for the temperature rise. These views were still held by Wollny in 1897 although Fry (1885), on the basis of Pasteur's work, considered that all the changes in the silo could be attributed to the activity of the plant cells which, under the conditions of restricted aeration, would respire to form incompletely oxidised compounds such as alcohol and acetic acid.

Fry's hypothesis had no experimental support until the publication by Babcock & Russel (1902). They reported that if maize was treated with chloroform, benzene or ether before packing, the 'silage' which resulted had a very low content of organic acid but was otherwise similar to normal silage. It was argued that the activities of the living plant cells and micro-organisms were inhibited by the antiseptics and that consequently, all the changes in such silage, except the small production of acid, must result from the/

the activity of plant enzymes. The origin of the acid produced in normal silage could not be deduced with certainty from such experiments, but additional evidence was presented to show that the acid-production was due to the metabolism of living plant cells. For example those conditions which prolonged the life of the plant cells under anaerobic conditions, eg. storage in hydrogen or nitrogen, resulted in an increased production of acid. No quantitative data or methods of analysis are given in this paper and its value would seem to be mainly in the novel approach to the problem.

A more detailed, but otherwise similar, investigation was reported by Russell (1908). He applied toluene to maize before ensiling in jars and obtained substantially the same results as Babcock & Russel, namely, that in such 'silage' there was no production of organic acid. In addition, however, it was shown quantitatively that the proteolytic changes were as extensive as in normal silage. It was concluded that this proteolysis was enzymatic in origin - the author specified plant enzymes - and the important point was made that proteolytic changes could obviously proceed even after the death of the plant cell. The formation of organic acids, which only took place if living plant cells and micro-organisms were present, was also considered to be due to the metabolism of the plant cells. One feels

more/

more confidence in the results of this paper than in those of any of the previous works mentioned. Experimental methods and analytical technique are detailed and some quantitative data are supplied. Based on these results the hypothesis as to the whole mechanism of the ensilage process, which Russell put forward, bears a surprising similarity to present views on the subject.

Further results obtained by ensiling crops in the presence of antiseptics were published by O.W. Hunter (1917, 1918). Alfalfa, maize and other crops were treated with antiseptics, with heat, and by wilting and packed into thermos fruit jars. Only in those cases where bacteria could multiply, eg. when the plants were packed without treatment or when they were heat-sterilised and re-inoculated with micro-organisms, was any rise in temperature recorded. It was inferred that the heat production in silage was mainly the result of microbial metabolism. In his later paper O.W. Hunter (1918) failed to detect any proteolysis in 'silage' prepared from alfalfa treated with chloroform. He, therefore, suggested that protein-breakdown in silage was mainly due to the activity of micro-organisms especially the lactic acid bacilli. A more likely explanation of Hunter's observation would be that the anaerobic flora, which he did not study, was responsible for the proteolysis.

C,A. Hunter/

C.A. Hunter (1921) produced some convincing results with silage prepared in milk bottles from Canada field peas and oats. A comparison was made between silage made from untreated material, from material treated with chloroform, and from heat-sterilised material which had been re-inoculated with silage juice. Bacteriological examination showed that the total number of bacteria was reduced to a low level by the chloroform in five days and no increase in acidity occurred. On the other hand the re-inoculated material acquired an acidity which was lower than , but of the same order of magnitude as in the untreated fodder. Thus bacteria appeared to be the principal agents for the production of acid. Protein-breakdown in the normal silage was, however, only equalled in the 'silage' prepared in the presence of chloroform and this proteolysis was, therefore, attributed to the activity of plant enzymes.

It appeared however, from the work of Gerlach, Gunther & Seidel (1927) that bacterial activity was in part responsible for ammonia production in silage. Thus treatment of grass with formalin completely inhibited the growth of micro-organisms and only a slight increase in ammonia occurred. On the contrary toluene failed to control the microflora and ammonia production was not hindered.

In a series of papers which are listed in one

of/

of them (Virtanen, 1936) the effect of acidifying green fodder with mineral acid before ensiling was reported. The main results were incorporated in a paper by Virtanen (1933). The proteolytic enzymes of plant cells and bacteria were found to be almost completely inhibited below pH4. Thus, if the initial pH of the fodder was reduced to 3.6 units only slow formation of soluble nitrogen compounds took place, mainly in the form of amino acids. The small quantity of ammonia-N which occurred was derived from amides but whether it arose as a result of plant cell or microbial activity is not clear for even at the low pH a metabolising microflora was present (Virtanen, 1937). The absence of any breakdown of amino-N to ammonia in such material, does, however, suggest that bacteria are normally responsible for this process.

Relatively few investigations have been made on the proteolytic enzymes of plants. Greenberg & Winnick (1945) in a review of the subject, list eleven plant proteases which have been obtained in a fairly pure state and these were derived from latex-producing plants or from fruits. Tracey (1948) reviewed the few papers which were available on the proteases of non-latex producing green plants and reported his own work on the protease of tobacco leaves and of other plants including cocksfoot. The tobacco enzyme had its maximum stability and activity at/

at about pH5 and was papain-like in properties. It did not appear to differ in properties from the protease of cocksfoot. At pH4 the activity of the enzyme was reduced by approximately 50%. A considerable variation in the protease content of the plants, depending on the cultural conditions and time of harvesting, was observed. Thus in a later report (Holden & Tracey, 1948) it was found that increased applications of nitrogen fertilizer reduced the protease content of tobacco leaves. The reverse effect was obtained when the application of phosphate was increased.

Conclusions based on the results of experiments involving the addition of antiseptics to plant material would seem to be unreliable for a number of reasons. Firstly, it would seem unlikely that any of the effects due to the living plant cells and micro-organisms could be obtained by such means for both are destroyed or inhibited by the treatment.. The only possibility is that some information might be obtained about the activity of the endo-enzymes of the plant cells. The enzymes present, after treatment of the fodder with antiseptic, will, however, include not only plant enzymes but also those of the initial microflora of the plant. Consequently any chemical changes, which take place in such silage, cannot with certainty be attributed only to plant enzyme activity. Again, since plant

respiration/

respiration is prevented owing to the death of the plant cells there can be no rapid establishment of anaerobic conditions in the 'silage' as is normally the case. Also the effects of the antiseptics on the different enzyme systems of the plant cells are unpredictable. Thus chloroform is known to inhibit peptidases (Sumner & Somers, 1947). In such experiments the unequal distribution of the inhibiting agent may result in false conclusions being drawn. This is very likely to occur when a non-volatile inhibitor eg. mineral acid is used. (Cunningham & Smith, 1939).

2. Investigations which have sought some correlation between the chemical and bacteriological changes in silage.

In such work a sufficiently high correlation is taken to indicate cause and effect. Thus Eston & Mason (1912) when examining large-scale experimental maize silage found a good correlation between total numbers of bacteria and the temperature and acidity of the mass. In their view, therefore, bacteria were the most important agents in the ensilage process. This example serves to indicate the danger of such deductions; it is reasonable to suppose that plant enzyme activity was responsible for the temperature rise which then resulted in increased bacterial action.

Similar/

Similar results to those of Esten & Mason (1912) were obtained by Hunter & Bushnell (1916) in silos containing lucerne and lucerne plus carbohydrate material. In both cases a close correlation was observed between total acidity and the numbers of bacteria belonging to the Bulgaricus group. It was inferred, therefore, that acid formation was due to bacterial activity.

A correlation between acidity and numbers of bacteria was again noted by C.A. Hunter (1921) in mashlum silage. In addition the production of ammonia increased concurrently with the numbers of acid-producing organisms, thus supporting the view of O.W. Hunter (1918) (see p. 5 of thesis) that these bacteria had some proteolytic action in silage.

Peterson & Fred (1920) inoculated unsterile and heat-sterilised maize with pentose-fermenting bacteria and found that, whether or not other organisms were present, these pentose-fermenters could bring about an acid fermentation of silage comparable to that obtained in normal silage. With maize silage on a large scale Peterson, Hastings & Fred (1925) observed that the occurrence of appreciable quantities of fermentation products coincided with the presence of large numbers of bacteria (over 10^9/ml. of sap.).

It seems improbable that conclusive results can be gained from experiments, such as those reported so far/

far in this section, which attempt to correlate chemical changes in silage with total numbers of bacteria. The aim should be to correlate any particular chemical change with the numbers of those bacteria which are known to be capable of effecting such a change in pure culture. The difficulty here is that only inadequate methods have existed for counting the different types of organisms in silage. The counting of anaerobes has usually been neglected for this reason.

The limitations of the conclusions to be drawn from bacterial counts were appreciated by Allen, Harrison, Watson & Ferguson (1937) who followed the chemical and bacteriological changes in grass silage made in test tubes and small silos. They attempted no detailed correlations although a statement, based on the high numbers of lactobacilli which were present throughout the incubation period, was made. They said (p.280) "Although, in the absence of precise knowledge as to the products of plant respiration it would be unwise to make a definite statement, it seems reasonable to assume that this lactic acid is largely, if not entirely, brought about by lactobacilli and coliform bacteria, particularly the former." Yet two of the same workers (Watson & Ferguson, 1937, p.I) state without evidence "Even after the exhaustion of this (oxygen) supply, intracellular respiration must/

must take place with the production of alcohol and certain of the simpler fatty acids of which acetic is the chief."

3. Other approaches.

Lamb (1917) made a novel attack on the problem in an endeavour to avoid the likely pitfalls introduced by the use of antiseptics. It was based on work by Rahn (1910) which suggested that enzymic changes could be differentiated from those dependent on microbial growth by the lag phase which is characteristic of the latter. Thus, when the rate of disappearance of substrate or the rate of appearance of products is illustrated graphically, the shape of the curves should indicate which of the two agents has been active. Lamb interpreted his results from this standpoint and arrived at the following conclusions with respect to maize silage:

(1) Acid production is mainly due to bacteria.

(2) Proteolysis is primarily caused by plant enzymes and later by micro-organisms.

(3) Alcohol, initially produced by plant enzymes, is later a result of yeast metabolism.

(4) Carbon dioxide is dependent on plant enzyme action.

(5) Heating of the silage is largely a result of microbial activity.

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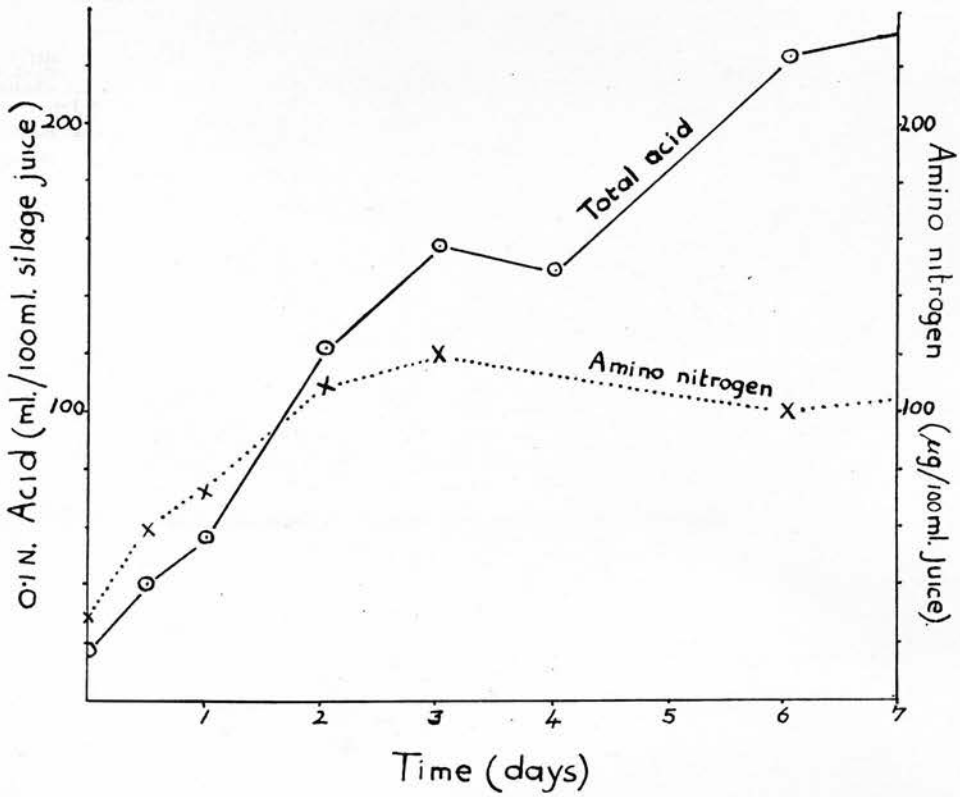


Fig. 1. Rate of proteolysis and of acid production in maize silage. (from data by Lamb, 1917).

A study of this interesting paper is accompanied by misgivings as to the validity of the deductions which are made. Two main points may be made here; the curves are often irregular and thus difficult to assign to the bacterial or enzyme class, and any attempt at differentiating between the two agents is impossible if both are responsible for a particular chemical change. The difficulty of interpreting the curves is illustrated in FIG. I which is constructed from the data obtained by Lamb in his experiments of Series 4. It is avowed from the shape of the graphs that the amino-N is produced as a result of plant enzyme activity and that acid production is a bacterial process.

Conclusions (4) and (5) appear to be theoretically incompatible. If the microbial activity results in little or no carbon dioxide the energy yield will be small as in a homolactic fermentation. A calculation, similar to that made by Woodman & Amos (1926), shows that for a rise in temperature of 25° , as recorded in Lamb's silage, the amount of sugar fermented would need to be about 15% of the fresh weight as against 3% actually utilised according to Lamb's figures.

A much more fundamental approach to the problem was selected by Trautwein (1928). His object was to prove conclusively whether or not lactic acid could be produced by the metabolism of germ-free maize plants/

plants which were packed in test-tubes. In brief germ-free maize seeds were planted singly, in large sterilised glass tubes, on nutrient medium and allowed to grow for 3 weeks under aseptic conditions. At harvest time each tube was sterilised externally by flaming and dropped into a sterile cabinet where a second worker, with well-cleaned hands, removed the plants and packed them into sterile tubes. After an incubation period of 5 months at room temperature the material was analysed for lactic acid using a modification of the method of Fürth & ~~Charnass~~ (1925). No lactic acid could be detected in excess of the small quantity present in the fresh maize plants (.03%) and it was concluded that the lactic acid in normal silage was not formed as a result of the metabolsim of plant cells.

The hazardous nature of this type of experiment is immediately obvious to anyone familiar with aseptic technique although checks on the sterility were made at various stages.

Summary of sections 1-3.

The results which have been discussed in the preceding sections of this introduction are so diverse that it was considered worthwhile to summarise the main conclusions in tabular form. (Table 1).

It/

Table 1. Summary of results discussed in Sections 1-3

Reference	Crop	Experimental approach	Agents responsible for production of				
			Proteolysis	Acid	Alcohol	Other products	Heat
Fry (1885).	-	-	Plant cells	Plant cells	-	-	Plant cell
Babcock & Russel (1902).	Maize	Plants + antiseptics	Plant cells	Plant cells	-	-	-
Russell (1908).	Maize	Plants + antiseptics	Plant cells mainly	Plant cells	Plant cells	Some fatty acids & amines by Bacteria	Plant cell
Hunter (1921).	Mashlum	Plants + antiseptics Plants + inoculation	Plant cells	Bacteria	-	-	-
Gerlach et al. (1927).	Grass	Plants + antiseptics	-	-	-	Ammonia by Plant cells	-
Esten & Mason (1912).	Maize	Chemical & Bacteriological	-	Bacteria	-	-	Bacteria
Hunter & Bushnell (1916).	Lucerne	Chemical & Bacteriological	-	Bacteria	-	-	-
Hunter (1917, 1918).	Lucerne	Plants + antiseptics	Bacteria	-	-	-	Bacteria
Lamb (1917).	Maize	Interpretation of graphs	Plant cells initially Bacteria later	Bacteria mainly	Plant cells initially yeasts later	-	Bacteria
Hunter (1921).	Mashlum	Chemical & Bacteriological	Bacteria	Bacteria	-	-	-
Peterson et al. (1925).	Maize	Chemical & Bacteriological	Bacteria	Plant cells initially Bacteria later	Plant cells initially Bacteria later	-	Bacteria
Trautwein (1928).	Maize	Sterile 'silage'	-	Not by Plant cells	-	-	-
Allen et al. (1937).	Grass	Chemical & Bacteriological	-	Bacteria mainly	-	-	-

It is seen that interest has been centred on the agents responsible for the main changes in silage, namely, proteolysis and acid formation. By 1925 there appears to have been a complete re-orientation of view as to the agents involved. Thus Russell (1908) attributed the main changes to plant enzymes with bacteria playing a subsidiary part, but in the following years more prominence was given to the bacterial population, until in 1925 we have Peterson et al. relegating the plant enzymes to a minor role. Since that time investigators have apparently had a greater appreciation of the difficulties involved and have been more cautious in presenting their views.

It is clear, as noted in section 1, that experiments involving the use of antiseptics are unlikely to provide conclusive results owing to the limitations of the approach. Again, the chemical and bacteriological analysis of silage after different periods of incubation, although capable of providing valuable data, has up to now been greatly handicapped by the lack of precise methods for counting the different species of micro-organisms.

Of all the approaches which have been attempted there is little doubt that the one most likely to produce conclusive results is that involving the preparation of silage from germ-free fodder. If this can be satisfactorily accomplished then all the chemical/

chemical changes which take place in the product may with certainty be attributed to the activity of the living plant cells or to their enzymes. This approach is the one which has been adopted in the experimental part of this thesis.

4. Relevant studies in Plant Physiology.

It is now established with reasonable certainty that plant tissue is capable of respiration not only in air but also under conditions of low or zero oxygen tension. (Stiles, 1946 and Goddard & Meuse, 1950). Such anaerobic respiration has been mainly studied with fruits and storage organs of plants and only in relatively few cases with leaf tissue. A feature of this work is the insufficient attention which is generally paid to the sterility of the material under investigation. Bearing in mind that micro-organisms may have a fermentation rate considerably higher than plant tissue the validity of some of the results may be questionable. This factor is, however, unlikely to be serious when tissue slices are being studied. (Turner, 1938 and Marsh & Goddard, 1939). Nevertheless no respiration studies have been made on sterile mature leaf tissue owing to the difficulties involved.

From the large amount of work done on the respiration/

respiration of plant tissue it seems reasonable to assume that, when fodder is packed into a silo, it will respire aerobically until the concentration of oxygen falls to a threshold value (the so-called extinction point of ~~an~~aerobic respiration) after which the tissue may respire anaerobically. It is, therefore, important to establish from the literature what are the likely products of such metabolisms and to what extent they contribute to the amount of proteolysis and acid formation in the silo. Particular attention will be paid where possible, to those investigations in which leaf tissue of gramineous plants has been studied.

Table 2. Respiration intensity of various plant tissues

Plant tissue	Respiration rate a)	b)	c)	Reference
Lupinus albus	73.2			Aubert 1892
Triticum sativum	291.0			Aubert 1892
Sunflower stem		0.31		Kidd et al. 1921
" leaves		0.90		Kidd et al. 1921
" inflorescence		1.13		Kidd et al. 1921
" leaves (36 days)		1.56		Kidd et al. 1921
" leaves (90 days)		0.45		Kidd et al. 1921
Triticum compactum				
" leaves (5 days)			85	Duff & Forward 1949
" " (13 days)			40	Duff & Forward 1949
" " (40 days)			28	Duff & Forward 1949

a) ml O_2/hr/g. fresh material

b) mg CO_2/hr/g. dry matter

c) mg CO_2/hr/100g. fresh material

Aerobic respiration. The intensity of respiration of any tissue may reasonably be assumed to depend on the activity of the protoplasm concerned. It is therefore not unexpected to find, as is shown in Table 2, that the respiration intensity of plant tissue depends on the species of plant and on the maturity and function of the tissue. Thus even the different quarters of a wheat leaf respire at different rates (Roberts, 1951).

Various external factors also influence aerobic plant respiration. From the point of view of silage making the effects of temperature, concentration of oxygen and carbon dioxide, and wounding would deserve the most consideration. For pea seedlings the highest initial intensity of respiration which could be sustained for several hours occurred at 35-40° (Stiles & Leach, 1932, p.41). At 45° although the rate was initially higher it dropped rapidly, but over a period of about 3 hr. the total CO_2 output was approximately equal to that at 35°. At 50° however the respiration intensity was initially much lower than that at 35° and it decreased rapidly with time.

If the respiration of grass responds in a similar way to temperature changes it would appear that the time required to establish anaerobic conditions in the silo will be decreased as the temperature rises to 40-45° but that at higher

temperatures/

temperatures a longer time might be necessary owing to the lower rate of respiration.

Wounding of the plant tissue has been known to increase the respiration intensity ever since the end of last century. More recently it has been studied by Audus (1935) who reported that rubbing of cherry-laurel leaves could increase their respiration intensity by 99%. Similar increases have been obtained with the wheat leaf (Roberts, 1951), the respiration returning, in a few hours, to a level which was just slightly above normal. No information can be found regarding the effect of more severe injury - eg. by mincing - on the respiration rate of leaf tissue.

The depressant effect of carbon dioxide on respiration is well established (Thomas, 1947, p.281) and is now widely utilised in gas storage of fruit and vegetables. It will not be further discussed. The effect of variation in oxygen concentration will be more conveniently dealt with in the section on anaerobic respiration.

It may be concluded from the evidence quoted above that the initial respiration rate of a grass crop, as it is packed into a silo, will be dependent on several factors among which the species of plant, its maturity, and the degree of wounding are likely to be important. Wounding may be a specially important factor when a cutter-blower or similar device is

employed/

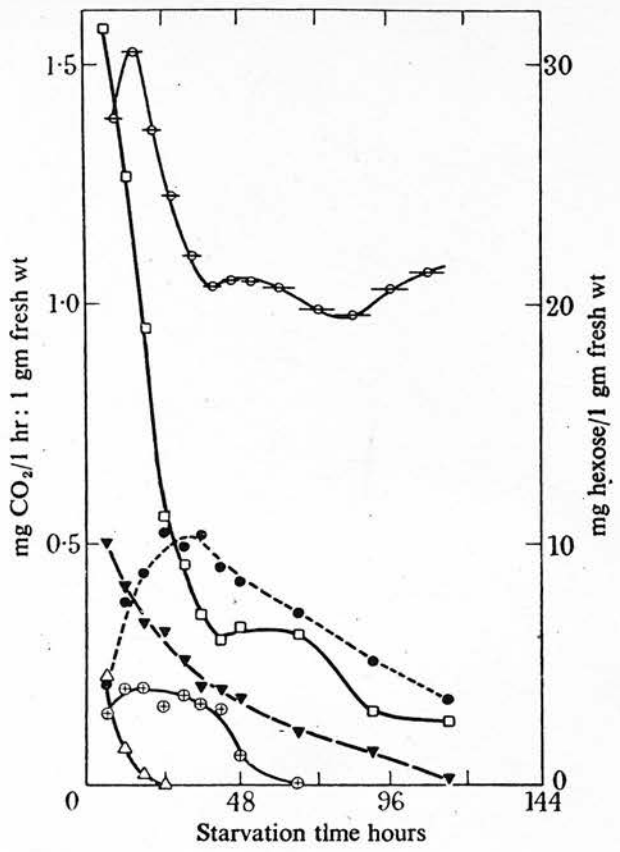


Fig. 2. *Carbohydrate exhaustion and carbon dioxide production.* △, *fructose;* ⊕, *fructosan;* ⊸, *mean carbon dioxide;* ●, *glucose;* □, *sucrose;* ▶, *starch*

From Yemm (1935).

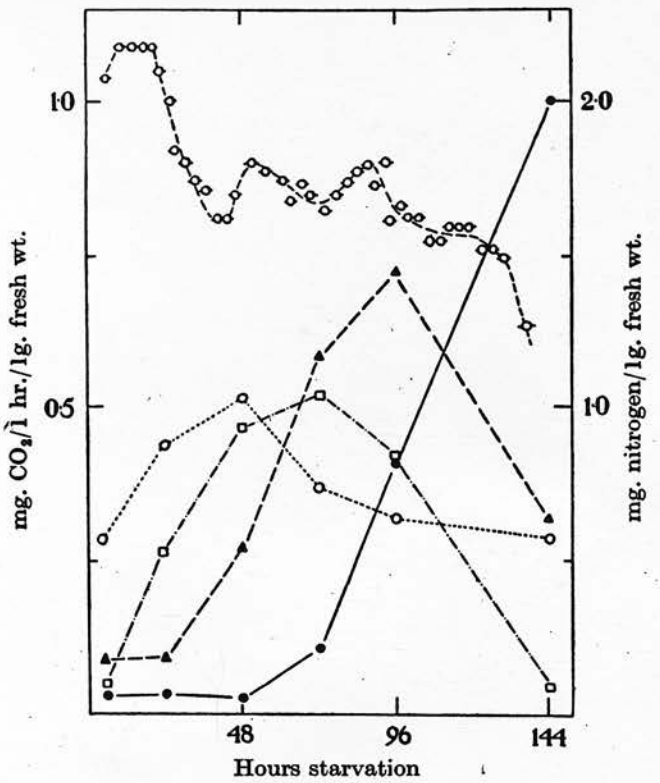


Fig.3. *Changes in amino, amide and ammonia fractions during starvation (Experiment XI), and mean rate of carbon dioxide production. Carbon dioxide production, -o-; amino nitrogen fraction, o; 2 × unstable amide (glutamine) nitrogen, □; 2 × stable amide (asparagine) nitrogen, ▲; ammonia nitrogen, ●*

From Yemm (1937).

employed in the filling process.

Another important aspect of aerobic respiration of plant tissue remains to be discussed. Within the last two decades various workers have investigated the metabolic changes associated with respiration in detached, starving leaves from which light is excluded. A study of their results suggests the possible chemical changes which might take place during the interval between cutting and filling or during an aerobic phase in the silo. Yemm (1935) studied the relationship between the concentration of carbohydrate, in the leaf of barley, and the intensity of respiration as measured by the production of carbon dioxide. Fig.2, represents the results from one experiment but the same general picture was obtained in a number of others. No simple relationship was found between the concentration of individual sugars (sucrose, fructose, glucose) and the rate of respiration, but the ratio $\frac{\text{rate of } CO_2 \text{ production}}{\text{total sugars}}$ remained constant up to 24 hr. suggesting that sugars were the only respiratory substrates during that period. Subsequently, the respiration rate was higher than could be accounted for by loss of sugar; this was considered to be due to the utilisation of an unknown substrate, possibly protein. Further work (Yemm, 1937, 1949-50) in which the nitrogen changes in darkened, detached barley leaves were followed, supported this view (Fig.3)/

(Fig.3). In the early period, up to 24 hr., respiration was accompanied by a fairly rapid proteolysis and a significant increase in glutamine content. Thereafter, an accumulation of asparagine occurred which reached a maximum during the yellowing of the tissue after about four days. Free ammonia was slowly formed after 48 hr. The glutamine production, even within 24 hours, was too high to be attributed to primary protein hydrolysis, suggesting that some amino acids were being oxidised to furnish ammonia for glutamine synthesis. Thus it appeared that some protein was being respired even in the early stage when plenty of available carbohydrate was present.

These conclusions were in general confirmed by Wood, Cruickshank & Kuchel (1943); Cruickshank & Wood (1945) working with starving leaves of Sudan grass and Kikuyu grass, but although they record a decrease in protein from the beginning of starvation, it does not become appreciable for 3-4 days if the leaves have a high content of sucrose.

Any changes in the organic acid content of plant tissue during aerobic respiration might also be important from the point of view of silage making for these acids are, in part, responsible for the 'buffering' of the tissue. Only in a few plants has the metabolism of organic acids been studied (Vickery & Pucher, 1940; Thimann & Bonner, 1950) notably/

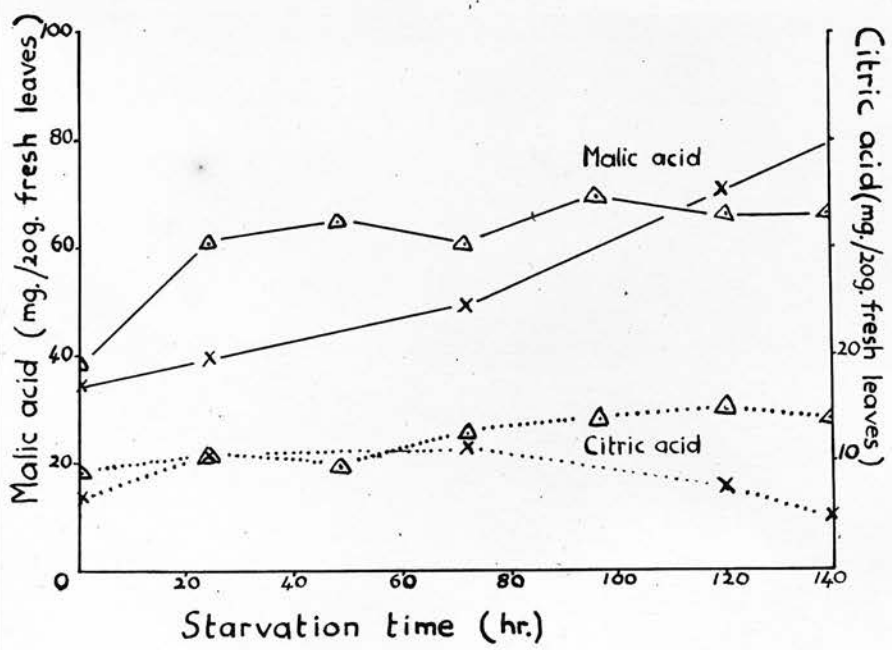


Fig.4. Changes in malic and citric acid contents of leaves of Sudan grass (x) and oats (Δ) during starvation in air (from data by Wood et al., 1943, and Cruickshank & Wood, 1945.).

notably in Tobacco, Rhubarb, Tomato, Begonia and in members of the family Crassulaceae which exhibit a diurnal variation in their total acid content. In the case of oats Pepkowitz, Gilbert & Shive (1944) found that malic acid was consumed at low oxygen tensions but in such investigations unknown acids comprise a large proportion of the total. Thus Yemm in unpublished data (Chibnall, 1939) found that malic and citric acids accounted for only about 50% of the total acidity of barley leaves. The change in concentration of these two acids during the starvation of detached leaves of Sudan grass and of oats was investigated by Wood et al. (1943) and Cruickshank & Wood (1945). As seen from Fig. 4, which has been constructed from their results, malic and citric acids tended to rise to a late maximum in the case of oats. In the case of Sudan grass, however, the citric acid reached an early maximum and then decreased. An interesting observation, from the point of view of the ensilage process, was that little or no increase in these acids occurred during starvation in nitrogen.

Succinic acid has been found in a large number of plant species (Pucher & Vickery, 1941) but appears to be present only in small amounts compared with malic, citric and oxalic acids, eg. in maize it represented 2.7% of the total acidity.

There is considerable evidence that the total

organic/

organic acid content of growing plants is higher if nitrate is used as fertilizer instead of ammonia salt. This has been shown to be true for grasses and clovers (Blackman & Templeman, 1940) and for oats (Pepkowitz et al.,,1944).

Although none of this data on aerobic metabolic changes has been obtained with pasture grasses of this country it is reasonable to assume that similar events take place during their aerobic respiration. Consequently it becomes important to estimate the duration of the aerobic phase in a silo. Peterson et al. (1925) by an analysis of the grass in maize silage estimated the period at about 5 hr; a figure of 3 hr. is reported by Tiemann & Rehm (1931) for lucerne silage. These figures are in general agreement with an estimation for grass silage in tubes reported in the experimental part of this thesis.

In farm practice, the filling of the silo may be intermittent and the material near the surface after each filling will have access to atmospheric oxygen. In such a case the metabolic changes discussed above may well be significant. Especially would this be the case where wilting of the crop is practised. For instance, in Yemm's experiments within 24 hr. half the sucrose and all the fructose had been respired and glucose had increased two-fold; in/

in addition a five-fold increase in glutamine and a 50% rise in amino-acid content had occurred.

It seems unlikely, however, that these respiratory changes will be of any great significance in laboratory tube silage which is sealed after packing. This may well cause difficulty in applying laboratory results to farm practice.

Anaerobic respiration. The anaerobic respiration of plants had been noted by the end of the 18th century but it was not until the end of the 19th century that Pasteur and his school established its general occurrence and showed that, besides carbon dioxide, alcohol was also produced. Since that time a large volume of work has been done on the subject, chiefly in connection with the respiration of seeds, fruits and storage organs (Stiles, 1946; James, 1946).

It is proposed to deal here mainly with investigations on members of the grass family and particularly with those aspects of their metabolism which might have some bearing on the ensilage process. It seems pertinent to emphasize again that, unless otherwise stated, the tissues used in the investigations were not free from micro-organisms.

Various workers have shown that anaerobic respiration may continue in the presence of low oxygen/

oxygen concentrations and is inhibited when this concentration reaches a critical value (the extinction point of anaerboic respiration). For maize seedlings the value is about 5% oxygen (Stich, 1891) and for barley seedlings about 2.5% (Forward, 1952).

When respiration takes place in the complete absence of oxygen the concomitant metabolic changes are not completely represented by the equation

$$C_6H_{12}O_6 \text{ --- } 2C_2H_5OH + 2CO_2$$

for if the ratio $\frac{\text{rate of alcohol accumulation}}{\text{" " } CO_2 \text{ production}}$ is determined for different plants the quotients obtained range from zero (potatoes) to 0.90 (apples) compared with a theoretical value of 1.04 (Stiles, 1946). This indicates that in some species anaerobic respiration is predominantly alcoholic in nature whereas in some tissue very little alcohol accumulates. In the latter case there is no satisfactory explanation for the production of the carbon dioxide, for the only other product which has been convincingly demonstrated is acetaldehyde (Thomas, 1947, p.288) and this occurs in amounts which are quite insufficient to account for low values of the quotient.

The alcohol seems clearly to arise from the activity of zymase for this enzyme complex has now been isolated from a large number of different plant tissues. In the case of barley sap the investigations/

investigations of James & Bunting (1941) and James, Heard & James (1944) have provided strong evidence for the existence of a phosphorylation sequence resembling the Embden-Meyerhoff scheme for yeast and muscle fermentation. On the basis of such work the assumption is now prevalent that aerobic and anaerobic respiration of plant tissue proceed along a common pathway up to and including pyruvate. Thereafter, the latter may then be oxidised aerobically to carbon dioxide, for instance by a tricarboxylic acid cycle, or under anaerobic conditions may be de-carboxylated and reduced to alcohol. Obviously other alternatives are possible; for example lactic acid might be the reduced product.

The capacity to respire anaerobically varies with the species of plant and the function and maturity of the tissue involved. Values obtained for the ratio $\frac{\text{anaerobic respiration}}{\text{aerobic respiration}} \left\{ I/N \right\}$ as measured by carbon dioxide production range from 0.25 for maize and 0.56 for wheat seedlings to 1.5 for ripening apples (Blackman & Parija, 1928; Leach, 1936). This quotient is, however, not easy to obtain accurately owing to the rapid fluctuation in I even after only a few hours under anaerobic conditions.

The respiratory response of plant tissue, when deprived of oxygen, appears to be one of two main types/

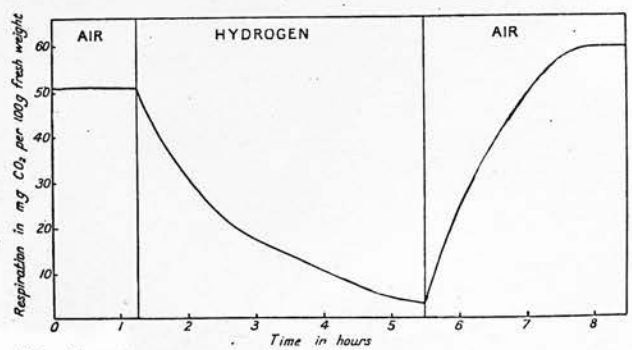


Fig. 5. -Graph illustrating the effect on carbon dioxide output of seedlings of *Sinapis alba* when changed from an atmosphere of air to one of hydrogen and *vice versa*
(*From data published by* Boysen Jensen)

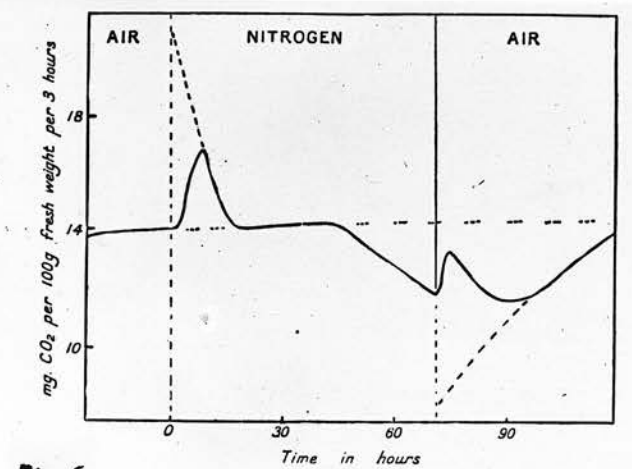


Fig. 6. –Graph illustrating the effect on carbon dioxide output of Bramley's Seedling apples belonging to the slow-ripening class, produced by change from an atmosphere of air to one of nitrogen, and *vice versa*
(*After* Blackman)

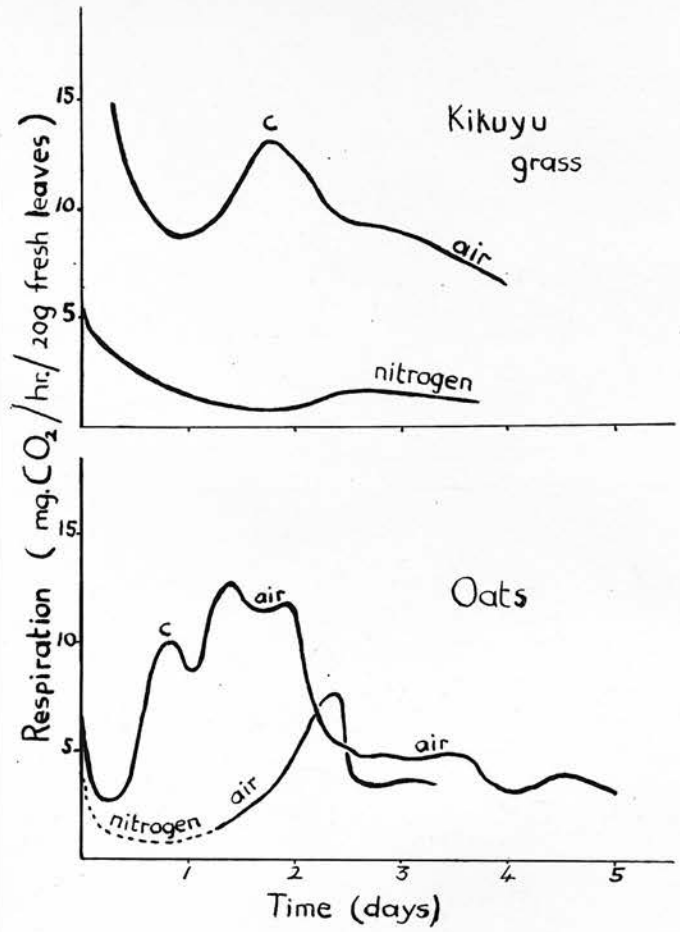


Fig. 7. Respiration of leaves of oats and Kikuyu grass (from data by Cruickshank & Wood, 1944.)

types. In the first class (Fig.5) a rapid decrease in intensity of respiration takes place and is maintained until the tissue dies. In the second class the decrease in rate of carbon dioxide production is preceded by an initial large increase of short duration (Fig.6).

Members of the Gramineae appear to belong to the first type. Wood et al. (1943) and Cruickshank & Wood (1944) constructed numerous respiration curves for Sudan grass, Kikuyu grass and oats, and from their data the graphs shown in Fig.7 have been compiled to illustrate the rapid decline in respiration which occurs under anaerobic conditions. Similar results were obtained for barley leaves (Philips, 1947). It would seem, then, that these grasses have little capacity for anaerobic respiration. Correspondingly, the metabolic changes associated with such respiration are likely to be comparatively small. Thus Cruickshank & Wood (1944) found that the rate of proteolysis and the accumulation of amino-acids was much lower in nitrogen than in air. In addition, no amides and no ammonia could be detected in nitrogen and the changes in citric and malic acids were insignificant. Another feature was the close correlation between respiration and utilisation of sucrose under anaerobic conditions whereas in air such correlation was/

was noted only in the very early period preceding the climacteric at C (Fig.7). It is, however, interesting to note that protein breakdown in nitrogen became appreciable if the tissue was extensively damaged.

Miller (1931, 1938) stated that under anaerobic conditions the end products of respiration of plant tissue "are carbon dioxide and a number of more or less complex compounds including ethyl alcohol, acetic, formic, oxalic, propionic, other organic acids, and probably molecular hydrogen." This statement was accepted by Watson (1939, p.20) who later (p.21) said that "in the silo the anaerobic respiration is the cause of the presence of large amounts of organic acids and of alcohol, though bacterial action confuses the issue somewhat." Miller, however, gave no references to original work in support of his statement which was greatly at variance with the view of Thomas (1947) outlined on p. 26 of this thesis. Because of these divergent opinions the possibility of the compounds mentioned arising from anaerobic plant metabolism. will be considered in more detail.

a) Alcohol.

From the preceding discussion (p. 26) it is taken as proved that plants under anaerobic conditions can produce alcohol in varying amounts.

In/

In particular the tissue of ripening fruit accumulates appreciable quantities when respiring anaerobically (Thomas & Fidler, 1941). The presence of alcohol oxidase in some plants (Ludwig, Allison, Hoover & Minor, 1940) suggests that in some cases alcohol may be an intermediate metabolite even under aerobic conditions.

The work of James et al. (1941, 1946) makes it probable that the necessary enzymes for alcohol production are to be found in members of the family Gramineae. One of the major points at issue, therefore, is to what extent the alcohol present in silage is likely to arise from anaerobic plant metabolism. Philips (1947) provided some data on this aspect, some of the results being obtained on tissue free from micro-organisms. She observed that the shoots of 4-day-old barley seedlings, which had been cultured aseptically, produced carbon dioxide at the rate of 9 mg./hr./20 g. fresh material and alcohol at 5.2 mg./hr./20 g. fresh material over an 8 hr. period in the absence of oxygen. The results were, however, quite different for older barley leaves which unfortunately were not sterile. Thus 10-day-old leaves produced alcohol at a rate less than 1 mg./hr./20 g. fresh material over an 8 hr. period and even this small production was doubtful owing to a large variation in the control value. It is interesting/

interesting to note that a much higher production (about 2 mg./hr./20 g. fresh material) was obtained over an 8 hr. period under aerobic conditions.

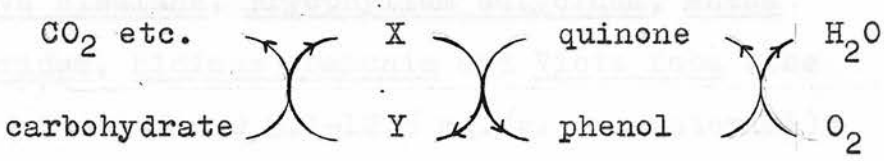
The alcohol content of silage averages about 0.3% of the fresh material (Watson, 1939, p.21).or 60 mg. per 20 g. fresh material. From the data presented above it seems unlikely that very much of this is the result of plant metabolism but it is not possible to draw any definite conclusions from the data available.

As previously noted (p. 26) the $\frac{\text{alcohol}}{CO_2}$ ratio suggests in some cases that other products besides alcohol must be formed. If a homolactic fermentation took place the value of the ratio would not be affected except in so far as the acidity produced might release the "bound" carbon dioxide of the plant tissue.

b) Lactic acid.

The presence of α-hydroxy-acid oxidases has been observed by Clagett, Tolbert & Burris (1949) in the leaves of a large number of plants. These authors studied, in particular, the oxidases from tobacco and barley leaves and found them capable of oxidising lactic and glycollic acids. The suggestion was made that such substrate-enzyme systems might be the whole or part of the unknown component/

component in the polyphenol oxidase respiration of plants as shown diagrammatically below.



If this were so it is conceivable that lactic acid might increase in amount if the polyphenol oxidase became inhibited e.g. under anaerobic conditions. At any rate the widespread existence of a lactic acid oxidase is suggestive that lactic acid is a common intermediate of plant metabolism. James & Cragg (1943) had earlier noted that glycollic, lactic and tartaric acids caused an increased oxygen absorption when added to barley juice, the effect being particularly vigorous in the case of lactic acid. They could, however, find no evidence of a polyphenol oxidase system in barley, but consider that the α-hydroxy-acids serve as donators to an ascorbic acid oxidase system.

Earlier reports of the occurrence of lactic acid in plant tissue were critically examined by Franzen & Stern (1921) who confirmed its presence in Opium, Ricinus seeds, and in the fruit Tamarind, and also added their observation that it accumulated in raspberry leaves. Schneider (1941) examined a number of other species and found its occurrence/

occurrence more widespread than had been thought, e.g. in the leaves of Rubus sp., Lactuca sativa, Agava sisalana, Bryophyllum culycinum, Rheum hybridum, Ricinus communis and Vicia faba (the latter contained 9.1-12.5 mg./g. dry material). It was noted that a quick rise in lactic acid concentration took place during the germination of maize, bean and castor oil seed, but an equally sharp fall resulted when the root had penetrated the testa. An interesting observation was that germination in nitrogen did not affect the magnitude of this change in lactic acid content.

Potato tubers, which produce little alcohol when respiring anaerobically (p.26), are now known to produce lactic acid during the first twelve days in nitrogen. Saifi & Barker (1939) obtained a maximum of about 0.2% of the fresh weight over this period.

The failure of Trautwein (1928) to detect any lactic acid in maize "silage" prepared from microbe-free plants has already been mentioned (p. 13). Philips (1947) recorded an increase of 9-27 mg./20 g. of 4-day-old barley seedlings after 6 hours in nitrogen. However, leaves from 10-day-old plants showed no increase after the same incubation period in nitrogen.

c) Other organic acids and hydrogen.

The writer has found no evidence that starving leaves metabolising in the absence of oxygen accumulate other organic acids including acetic and propionic acids, as suggested by Miller (1931, 1938). Nor can any evidence be found that anaerobic respiration of plants is capable of producing hydrogen.

Summary of section 4. The evidence available suggests that the aerobic metabolism of plant cells, which occurs between the time of cutting and the establishment of anaerobic conditions in the silo, may result in some proteolysis and formation of amide and ammonia, accompanied by a decrease in amount of available sugar. Some increase in organic acid content is to be expected during this phase and may have some effect on the buffer capacity of the fodder. The extent of these changes will be mainly governed by the duration of the aerobic conditions.

Under anaerobic conditions the metabolic changes due to plant cell activity are difficult to predict owing to the absence of conclusive data. The evidence suggests that alcohol may be produced and that the formation of some lactic acid is a possibility/

possibility. The extent of protein breakdown in the first few days may be dependent on the degree to which the plant tissue has been damaged during the packing process.

METHOD OF PREPARING MICROBE-FREE PLANTS AND PREPARING MICROBE-FREE 'SILAGE'

As already suggested in the introduction to this thesis (p. 16) it was considered that the only reliable method of differentiating between those changes in the silo which are due to plant cells and those due to micro-organisms would be the preparation of 'silage' from microbe-free plants. Any changes which take place after incubation could then with certainty be attributed to the activity of the living plant cells or their enzymes.

The technique of growing microbe-free plants is frequently used in agricultural bacteriology for testing the efficiency of the legume nodule organisms. It involves the following stages: a) sterilisation of the seed coat, b) sowing of the seed on a sterile medium and c) maintenance of sterility during growth. The last two steps usually present no difficulty as it is a common practice to grow the plants in tubes which are plugged with cotton-wool. Such a procedure was considered unsatisfactory for this investigation because a reasonable yield of plants was required and these, in some way, had to be packed aseptically into small silos.

It seemed that the most satisfactory arrangement would be to conduct all the operations, from sowing the/

the seed to ensiling the plants, in the same sterile chamber. In this way the chances of contamination of the plant material should be minimised.

Preparation of microbe-free seed

Most legume seeds have smooth coats which permit thorough disinfection by chemical treatment. On the other hand many of the common grass seeds, e.g. of rye-grass, are enclosed in large glumes which would be expected to prevent efficient penetration of germicide. From this point of view timothy-grass seed seemed to offer the best chance of successful treatment and it was chosen for the preliminary investigation. It had the additional advantage that, as far as could be ascertained, it was not known to associate with any endophytic fungus, which might be unaffected by a surface sterilisation.

Numerous germicides have been employed for seed disinfection (Fred, Baldwin & McCoy, 1932) and of these some form of hypochlorite was considered the best. It was preferred to mercuric chloride solution whose use results in the adsorption of the mercury salt on the seed coat. Complete removal of the salt by washing is therefore difficult and might lead to false conclusions.when testing for sterility on a nutrient medium and might have some effect on the/

the growing seedling. Calcium hypochlorite, however, if incompletely removed in the washing process, decomposes quickly to calcium hydroxide which is innocuous.

Preparation and application of hypochlorite solution. A concentrated aqueous solution of bleaching powder was prepared by shaking excess calcium hypochlorite (10 g. in 140 ml.) with distilled water in a stoppered flask and allowing to stand overnight. Before use on the following day a clear solution was obtained by filtration through paper.

The seed (about 8 g.) was placed in a sterile screw-top vial (1 oz.) and covered with absolute alcohol for 2 min. Two washes of sterile water were given to remove the alcohol. The saturated solution of germicide was now added until the vial was completely full and, with the screw-top slightly loose, the vial was placed in a vacuum desiccator. Evacuation of this vessel enabled air bubbles to be detached from the seed, an important factor in permitting efficient contact of seed and germicide. Various contact times were tried. Four washes with sterile water completed the treatment.

Test for absence of micro-organisms. Some workers (Ash & Allen, 1948) consider it impossible to effect a complete kill of the organisms associated with seed/

seed, possibly because of the difficulty of efficient penetration of the germicide into the hilum scar and micropyle. Thus seed which was apparently sterile on the surface was found by these workers to liberate micro-organisms on crushing. To safeguard against this possibility sterility tests were conducted as follows.

About 20 seeds after treatment were allowed to germinate on sterile nutrient agar at 22° (7 days) and the plates were examined for contaminants at the end of the incubation period. About 20-30 seeds were also transferred by a spoon to a tube of sterile nutrient broth. On the following day duplicate quantities of 5-10 seeds were transferred to fresh broth and crushed with a sterile glass rod. All tubes were incubated at 22° for 3 weeks and, in the absence of visible growth, the broth was streaked on nutrient agar slopes and incubated at 22° for 7 days.

Germination test. A sample of the treated seed was incubated at 22° for 7 days on moist filter paper in a petri dish, to determine if the treatment had affected the germination.

Exp. 1 The seeds were in contact with germicide for 1, 2 and 3 hr. respectively. All samples were found to be grossly contaminated when plated on agar. No broth cultures were prepared.

Table 3. Effect of calcium hypochlorite treatment and various pre-treatments on the germination and microflora of timothy seed.

Exp. No.	Pre-treatment Alcohol min.	Pre-treatment Conc H_2SO_4 min.	Hypochlorite Contact time	Sterility Test Agar	Sterility Test Broth	Germination %
1	2	-	1 hr.	All badly	not-done	-
	2	-	2 hr.	Contaminated	"	-
	2	-	3 hr.		"	-
2	2	5	1 hr.	Sterile	"	0
	2	10	"	"	"	0
	2	15	"	"	"	0
	2	5	2 hr.	"	"	0
	2	10	"	"	"	0
	2	15	"	"	"	0
	2	5	3 hr.	"	"	0
	2	10	"	"	"	0
	2	15	"	"	"	0
3	-	1	1 hr.	"	Sterile	25
	2	1	"	+	"	25
	-	2	"	Sterile	"	0
	2	2	"	"	"	0
	-	1	3 hr.	"	"	24
	2	1	"	+ +	"	30
	-	2	"	Sterile	"	0
	2	2	"	"	"	0
4	-		3 hr.	"	"	85
	2		"	"	"	80
	-		6 hr.	+	"	20
	2		"	Sterile	"	15
	-	-	10 hr.	"	"	10
	2	-	"	"	"	0
	-	-	24 hr.	"	"	0
	2	-	"	"	"	0

\+ 1 mould colony per plate

++ 2 mould colonies per plate

Exp. 2 The pretreatment with alcohol was replaced by an immersion in concentrated sulphuric acid to burn off superficial organic material and thus to improve the efficiency of the germicide. Immersions of 5, 10 and 15 min. were tried, the seed being immediately washed in copious amounts of water before the application of hypochlorite. As seen from Table 3 contamination was slight but no seeds germinated.

Exp. 3 Shorter exposures to concentrated sulphuric acid were tried, both with and without the alcohol treatment. The seed in all cases was microbe-free by the agar and broth tests but again germination was poor.

Exp. 4 The unsuitability of the acid treatment made it necessary to pursue Exp. 1. Consequently contact times of 3 hr. and over were investigated. Previous experience had shown that it was not a simple matter to rid the seed of all attached air bubbles and in this experiment evacuation and refilling of the desiccator was repeated many times with frequent gentle shaking. As is seen from Table 3 the seed was now satisfactory even after the 3 hr. treatment, both from a germination and sterility point of view. Since a decrease in germination percentage took place with increased contact/

contact times, the 3 hr. treatment was selected as the best.

The alcohol treatment was not essential for the production of complete sterility but it facilitated the wetting of the seed, thus greatly assisting the removal of air-bubbles.

Further experiments showed that the result obtained in Exp. 4 could be repeated and in fact satisfactory results were obtained even with a 1 hr. contact time if care was exercised to remove air bubbles completely.

One disconcerting aspect of the results shown in Table 3 was the occasional appearance of one, or sometimes two, mould colonies. Since no corresponding growth took place in the broth test it was felt justifiable to assume that they were aerial contaminants picked up during the plating procedure.

Drying of the seed after hypochlorite treatment.
As will be appreciated later, when the design of the growth-chamber is discussed, it was desirable that the microbe-free seed should be in a dry condition. This also had the advantage that batches of treated seed could be prepared, tested for the absence of micro-organisms and stored until required.

Exp. 5 Seeds were treated, as previously described, with/

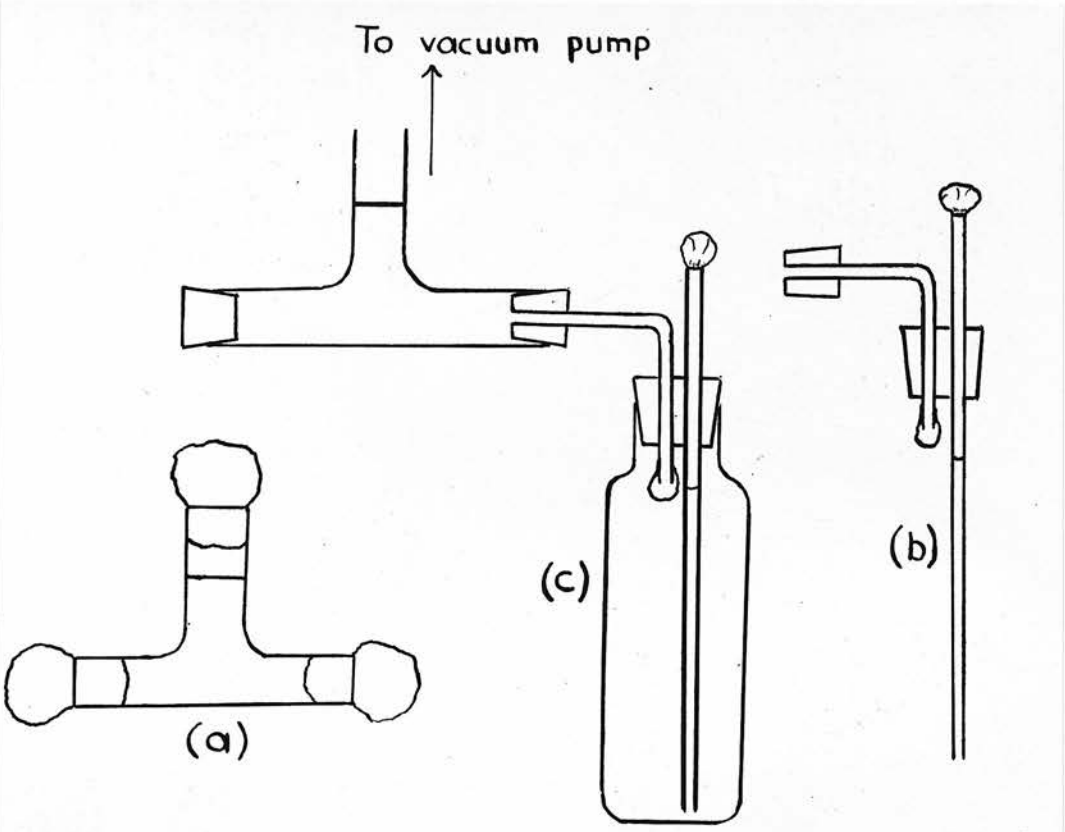


Fig. 8. Apparatus for drying microbe-free grass seed.

with alcohol (2 min.), calcium hypochlorite solution (3, 6, 24 hr.) and four water washes. They were then dried as illustrated in Fig.8. The glass T-piece (a), with one limb fitted with rubber tubing and the three orifices plugged with cotton wool, and the rubber stopper assembly (b), one of whose glass tubes served as an air filter, were sterilised separately in steam at 1½ atm. pressure. The lower stopper of assembly (b) was then fitted aseptically into the vial containing wet, microbe-free seed; the air-filter tube nearly touched the bottom of the vial when the stopper was fitting tightly. The upper stopper of assembly (b) was now pushed tightly into one limb of the T-piece (Fig.8c) and, if required, a second vial unit could be similarly attached to a second limb. Alternatively this limb could be closed by a sterile bung. The third limb of the T-piece was now connected by the rubber tubing to a silica-gel drying-tube attached to a vacuum oil pump. The latter was started after surrounding the vials with a water bath at 45°. In this way sterile air was drawn over the seeds and drying was effected in about 1 hr. On completion of the process the rubber stopper assembly was removed from the vial and a sterile screw top was fitted. Tests were then carried out, as before, for the absence of micro-organisms and for the percentage germination (Table 4).

Table 4. Effect of drying timothy seed after treatment with calcium hypochlorite solution

Exp. No.	Treatment Alcohol	Hypochlorite	Sterility Test Agar	Broth	Germination %
	2 min.	3 hr.	sterile	sterile	85
5	2 min.	6 hr.	"	"	20
	2 min.	24 hr.	"	"	1

It was concluded that a 3 hr. treatment with saturated calcium hypochlorite solution, followed by drying under vacuum at 45°, was capable of producing dry, microbe-free timothy seed without seriously affecting its germination properties. This result was repeatedly confirmed in subsequent work when numerous batches of seed were prepared for use in the growth-chamber.

Design of growth-chamber

When considering the type of growth-chamber to be employed for the growth of microbe-free timothy grass, a number of important requirements had to be taken into account. They were:-

a) The whole assembly should be capable of being autoclaved to ensure complete sterility.

b) The top portion of the chamber must be transparent to provide sufficient illumination for the growing plants.

c) Facilities should be provided for sowing the seed and harvesting and packing the plants without introducing contaminants.

d) It should be possible to provide a continuous, sterile, air-stream through the chamber and a sterile water-supply for the plants.

Requirement a) was satisfied by making the overall size of the assembled chamber 48 x 33 x 51 cm. These dimensions were such that it could just slip into the largest autoclave which was available.

It was more difficult to satisfy requirement b). The dimensions were unfortunately outwith the range of the domestic oven-ware in common supply and although a large number of glass manufacturers were asked to make a suitable top for the apparatus none would undertake the work, presumably because of the cost of making the mould. No assistance appeared likely from the plastics field for it appeared that the colourless, transparent products were thermoplastic and would not withstand autoclaving under pressure. Accordingly cellophane was considered. It was

essential/

essential that the material chosen should be impervious to water in order to provide an efficient barrier against micro-organisms. Accordingly, a strong, water-proofed cellophane, known in the trade as "diaphane", was selected for test. It proved capable, in preliminary trials, of withstanding steam at 2 atmospheres for 1 hr. without its water-repellent properties being impaired and without any serious reduction in tensile strength. The treatment did make the material more brittle. To support the cellophane a stainless-steel framework (Plate 1) was made of ½in. metal strip. The four sides and top were covered with one sheet of the material which was stuck to the metal at the lower edge, and sealed at the folds, with cellulose tape.

The lower half of the chamber (Plate 2) was also constructed of stainless steel. Its dimensions were such that the covered top fitted neatly into it with the "legs" of the top resting on metal projections in the corners of the base-part (Plate 3). In the front of the latter, two, circular holes, of approximately 4in. diameter, had been left and two circular rims of steel had been soldered in position. To these rims long, jointless, rubber gloves* could be attached and the operator was thus able to manipulate/

* Supplied by E. Draper, All British Works, Northampton.

manipulate inside the chamber without fear of introducing contaminants from the hands (Plate 4). This principle has been much used in the raising of germ-free animals (Reynier, 1943). The base-part was also provided with three, small inlets of $\frac{1}{2}$in. steel tubing. The side inlet (Plate 2) was inclined at about 45°. It projected a small distance into the interior of the chamber and served, as described later, for the introduction of the dry, microbe-free seed and of the water for the growing plants. The remaining two inlets were situated in the rear side of the base-part and are not visible in the photographs. To each was connected a cotton-wool filter, one serving to filter the incoming air and the other guarding the exit against a possible "suck-back".

The setting-up and sterilising of the growth chamber.

The description which follows applies to the initial experiments which were carried out. Reference will be made to any modification in technique when the experiments are reported in detail.

All the equipment, necessary for sowing of the seed and for the growth, harvesting and packing of the plants, was placed in the bottom of the base-part. It consisted/

consisted of the following items (Plate 2):

a) Crystallising dishes (9 cm. diameter) filled with acid-washed sand (Appendix 1). Each dish had a $\frac{1}{4}$in. hole drilled in the base and rested, on a triangle of glass rod, in a slightly larger dish. Drainage was therefore possible. The sand was saturated with a nutrient solution (Appendix 2) which had previously given satisfactory growth of timothy in sand culture.

b) Three vials filled with concentrated nutrient solution. This represented a reserve supply of nutrients if required.

c) Stainless-steel scissors and forceps. These were placed in a boiling tube plugged with cotton-wool so that they would keep as dry as possible.

d) A 150 ml. beaker containing,

1) three test-tubes of sterile nutrient broth,
2) one cotton-wool swab,
3) two 6 x $\frac{3}{4}$" test-tubes (one spare) into which the grass was finally to be packed,
4) one 5 x $\frac{1}{2}$" test-tube which was used to pack the fodder into the larger tube,
5) a sintered glass-mercury valve, the outlet being plugged with cotton-wool and the lower end carrying a rubber stopper which fitted the $\frac{3}{4}$" silo tube (Plate 5).

e) One small crystallising dish which fitted below the side inlet and served to collect the seed when it/

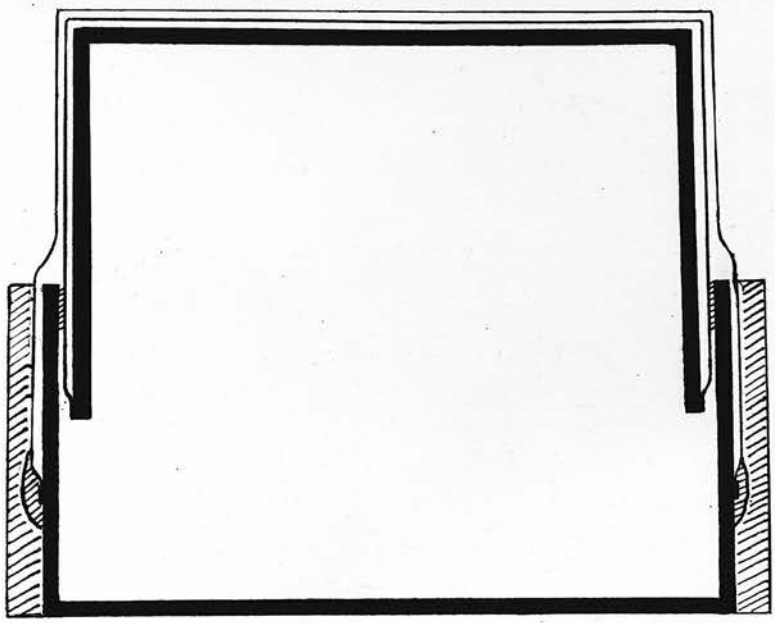


Fig.9. Transverse section of growth chamber showing positions of cellophane (single line) and cotton wool (hatched).

it was introduced.
f) A length of rubber tubing one end of which could be attached to the inner end of the side inlet, and a good supply of cotton-wool to be used for wrapping the silo-tube after packing and sealing.

The glove-holes of the base-part were initially covered with paper, the top of the base-part was similarly covered and the outer openings of the inlets were all plugged and wrapped in cotton-wool and paper. Autoclaving was carried out in steam at 2 atmospheres for 1 hr. to ensure complete sterility of the sand. The autoclaved base-part was then allowed to cool down to room-temperature in the autoclave. On withdrawing it, the paper covering was removed and the upper framework, covered with cellophane, was immediately placed in position with a cotton-wool packing forming a joint between the two (Fig.9). A second layer of cellophane was now wrapped over the frame so that its lower edge, which was stuck with cellulose tape to the base-part, was well below the cotton-wool packing. The cellulose-tape joint was protected by a two-inch wide, cotton-wool wrapping. The paper-covering was next removed from the glove-holes, a circular band of rubber made from bicycle inner-tubing, was slipped over each projecting rim and finally the gloves were fitted and secured with cotton-wool and string (Plate 4). The rubber bands/

bands prevented direct contact between gloves and metal which might have caused a deterioration in the strength of the gloves during sterilisation.

The assembled chamber was now completely wrapped in paper, to protect the cellophane during handling, and sterilised in the autoclave at 2 atmospheres momentarily. It was allowed to cool to room temperature in the autoclave

Preparation of sterile air filters and water supply.

The air filters were constructed from soft glass-tubing (1¼in. diameter), the inlet filter having a length of 12in. and the outlet filter 4in. Cotton-wool was packed firmly into the inlet filter; about 40g. of wool was used. The outlet filter was packed with glass-wool in the lower half and cotton-wool in the upper half. It was hoped that, if any condensation of moisture took place in the outlet filter, this arrangement would prevent wetting of the cotton.

To one end of each filter was attached a short piece of thick rubber tubing which would make the joint between filter and metal inlet. The rubbered end was wrapped in cotton-wool and the whole then sterilised at 2 atmospheres momentarily.

A 10 litre pyrex bottle served as a reservoir for the water supply. A rubber bung, which made

a/

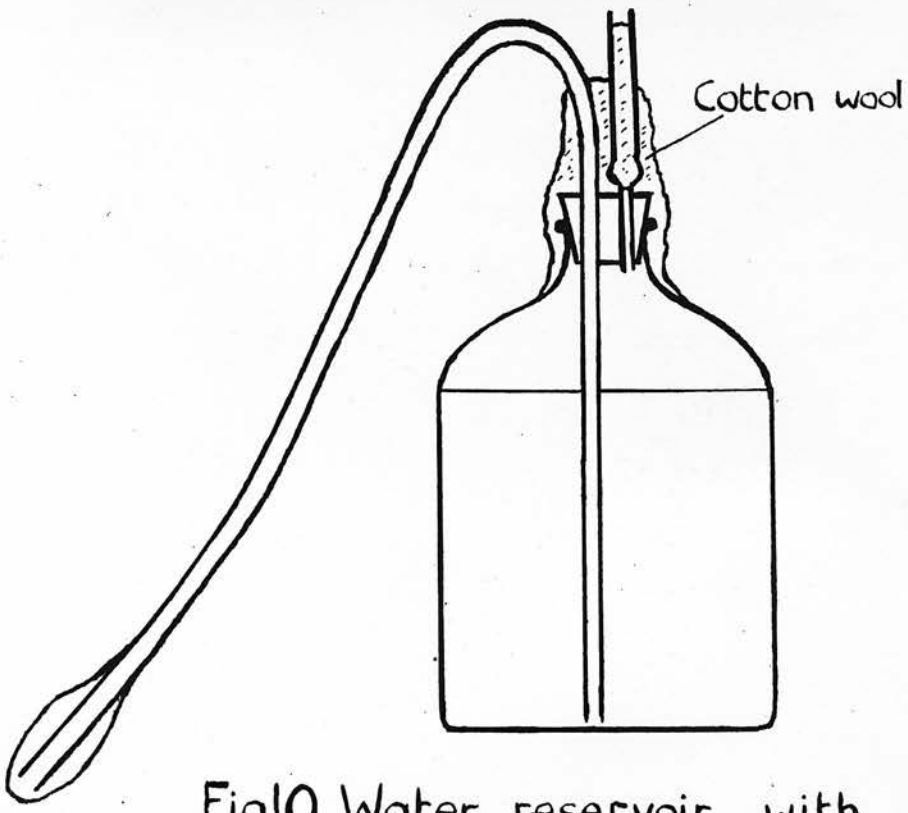


Fig.10. Water reservoir with air filter and siphon.

a tight joint with the mouth of the bottle, was fitted with a long glass tube, and with a cotton-wool air filter which was 6in. in length. To the glass tube was attached a length of stout rubber tubing whose free end was wrapped in cotton-wool and paper. The bung assembly was sterilized in paper at 2 atmospheres. Approximately 9 litres of water was sterilized separately (2 atmospheres for 1 hr.) in the bottle, which was temporarily plugged with cotton-wool. On cooling, the bung was screwed home tightly with flaming (Fig.10) and the joint securely wound with cotton-wool.

Attachment of water-supply and air filters

The paper and cotton-wool wrapping was quickly removed from the side inlet, the plug was taken out and, with flaming, the mouth of the vial containing microbe-free timothy seed, was quickly fitted over the orifice of the inlet (Plate 6). A sterilised metal plate was usually slipped over the inlet, prior to the removal of the plug, to protect the cellophane during the flaming process. The vial, which fitted tightly over the inlet, was now rotated and tapped to induce the seed to run into the chamber where they collected in the small crystallising dish which had been placed in position.

Immediately the vial had been emptied, the paper and cotton-wool covering was removed from the rubber-tubing attached to the water reservoir and, after withdrawing the vial, the tubing was pushed firmly over the inlet with flaming. A tight wrapping of cotton-wool served to protect the new joint (Plate 7).

In a similar way the rubbered ends of the air-filters were attached to the two rear inlets and the air-flow was started. The air was supplied by a small electric air-pump and a constant pressure was maintained on the filter by a pressure stabiliser similar in construction to the arrangement described by Bartholomew and Broadbent (1949). The rate of air flow through the filters was adjusted, by the insertion of a small piece of capillary tubing in the air-line, to about 4 litres per hour. This rate was found to produce a very slight positive pressure in the chamber, which might be an important factor in maintaining asepsis, and was considered to provide an ample supply of carbon dioxide for the growing plants.

As a final precaution the lower half of the completed assembly was wrapped with cotton-wool (Plate 7).

Sowing of the seed

The rubber gloves were liberally coated with talc/

talc and carefully fitted on the hands of the operator who sprinkled the seeds from the crystallising dish on the surface of the sand. This was not an easy process owing to the difficulty of reaching to certain regions of the chamber without straining the gloves.

Watering of the plants

After the sowing of the seeds had been completed the rubber gloves were used to attach the rubber hose, which was inside the chamber, to the inner end of the side inlet.

When the plants were established they were watered at about weekly intervals. The water was siphoned from the reservoir and distributed, via the hose, over the surface of the sand until excess water began to run through into the outer dishes. The siphon could be broken by lowering the reservoir below the level of the water inlet.

Illumination of plants

The details of the illumination employed will be given when each experiment is being considered separately. It will, however, be appreciated that only two crops could be expected during the spring and/

and summer season, taking into account the time required in preparing the chamber and analysing the grass and silage. Consequently, it was convenient to obtain a third crop when possible, during the autumn and winter season, by supplementing the daylight with artificial light. The latter was obtained from a 400 w. high-pressure mercury vapour bulb fitted horizontally into a parabolic reflector. The distance of the bulb from the reflector was just less than the focal length of the parabola, so that slightly divergent light fell on the chamber. The target distance was about four feet and the light intensity at this distance was approximately 300 ft. candles. After passing through two layers of cellophane the light was reduced in intensity by about 15%.

It was also considered worthwhile to investigate the possibility of growing the plants using artificial light only. In this way a greater control of growth conditions should be possible and the composition of the crop should be reproducible from one experiment to another. The mercury vapour lamp resulted in good growth of timothy in a small preliminary trial and it was decided to grow a crop inside the chamber with this illumination only. The spectrum of the light from this lamp is however, deficient in red light when compared to sunlight and the red end of the/

the spectrum is known to be especially important in photosynthesis.

Reference to lamp data (Lighting service bureau, 1950) made the following calculation possible. The total output of a 400 w. H.P.M.V. lamp is about 400 x 36 lumens of which only 1% or 144 lumens consists of red light (6,000 - 7,000 A^{o}). For natural light of the same intensity about 15% or 2,100 lumens would be made up of red light. Consequently about 2,000 lumens of red light are required to make up the deficiency in the quality of light from the H.P.M.V. lamp. This deficiency could be made up by use of a 300 w. tungsten lamp giving a combined light source of 18,000 lumens.

The combinations of tungsten and mercury vapour lamps which were actually employed are discussed later.

Harvesting of the grass and the preparation of 'silage'

When the yield of grass was estimated to be sufficient to provide enough material for analysis and for making into 'silage' the rubber gloves were fitted on to the operator who carefully pushed his hands forward into the cabinet, being careful to avoid laying the crop. The scissors were used to obtain/

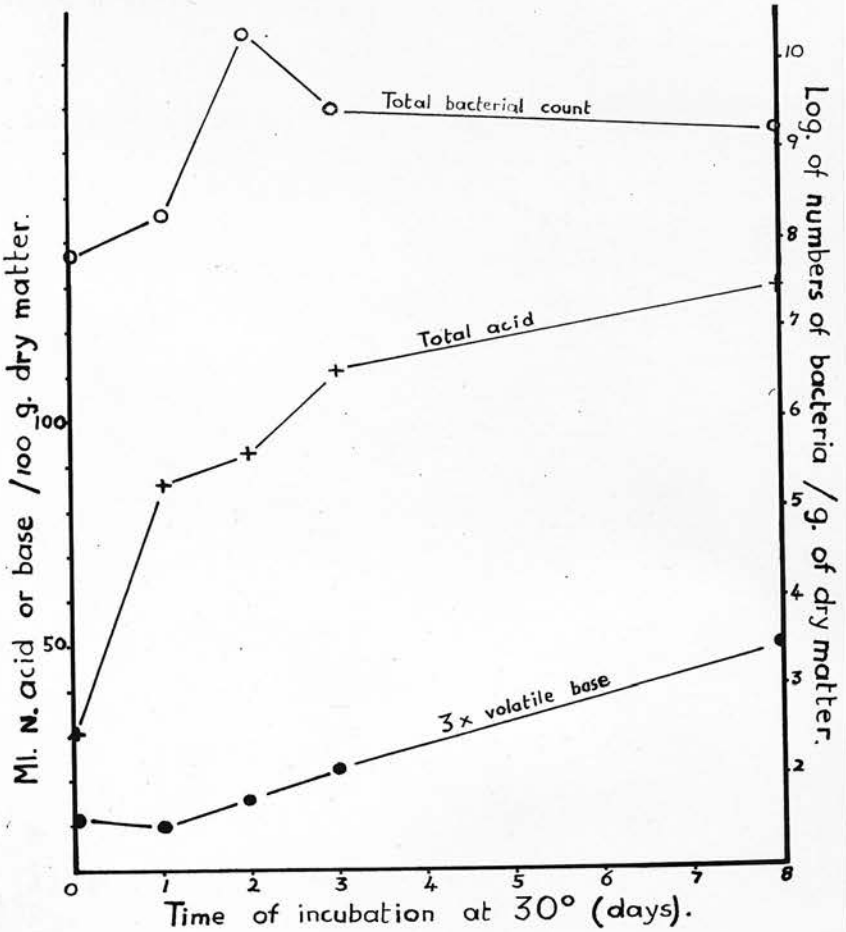


Fig.10b. Rate of chemical and bacteriological changes in grass silage made in test tubes.

obtain a few snippings of grass which were dropped into one of the tubes of broth. Then using both hands, about half the crop was sheared and placed by hand into the emptied beaker. Form here it was packed into the $\frac{3}{4}$in. tube, without further cutting, using the $\frac{1}{2}$in. tube as a packing-stick. The rubber bung, carrying the mercury-valve was then screwed in tightly and the joint wrapped with cotton-wool. Finally, samples of sand from the dishes were inoculated into the remaining two tubes of broth.

The cellophane covers were next torn off and the steel framework removed. This allowed the grass, which was left, to be harvested without aseptic precautions and taken to the laboratory for analysis. Alternatively, if quantitative data on the number of possible contaminants was required, the grass could be harvested and packed into the beaker before dismantling the chamber.

The packed silo-tube was incubated at 30°C for 7 days. This period was chosen for two reasons: previous experience had shown that the organisms which multiply in grass silage are still at about their maximum numbers at this time and any contaminants should, therefore, be easily detected; and chemical changes in grass silage are appreciable after such an interval (Fig. 10b).

METHODS OF CHEMICAL ANALYSES

The methods which have proved useful in the past for the chemical analysis of silage have been mainly based on the work of Foreman (1920, 1928) who showed that, in solutions containing approximately 85% alcohol by volume, most of the common amino-acids, and the salts of ammonia and amines, can be titrated quantitatively with alkali using phenolphthalein as indicator. Extension of this procedure (Woodman, 1925; Smith, 1938; Watson 1939, p.273) allowed a separation of the total acidity of the silage extract into the salts of volatile bases, amino-acids, non-volatile and volatile acids. The individual volatile acids could then be estimated by some distillation procedure (Dyer, 1917; Wiegner, 1926).

Although the application of these methods provides a useful picture of the quality of silage they are tedious in practice, and require much laboratory bench space, because of the number of distillations involved and the necessity for concentrating large volumes of distillate. These defects have been borne in mind when developing the methods described below. In general micro-methods have been favoured as they enable small quantities of grass or silage, as would be obtained from a crop from the growth-chamber, to be dealt with satisfactorily. Nevertheless some of the procedures described may be useful/

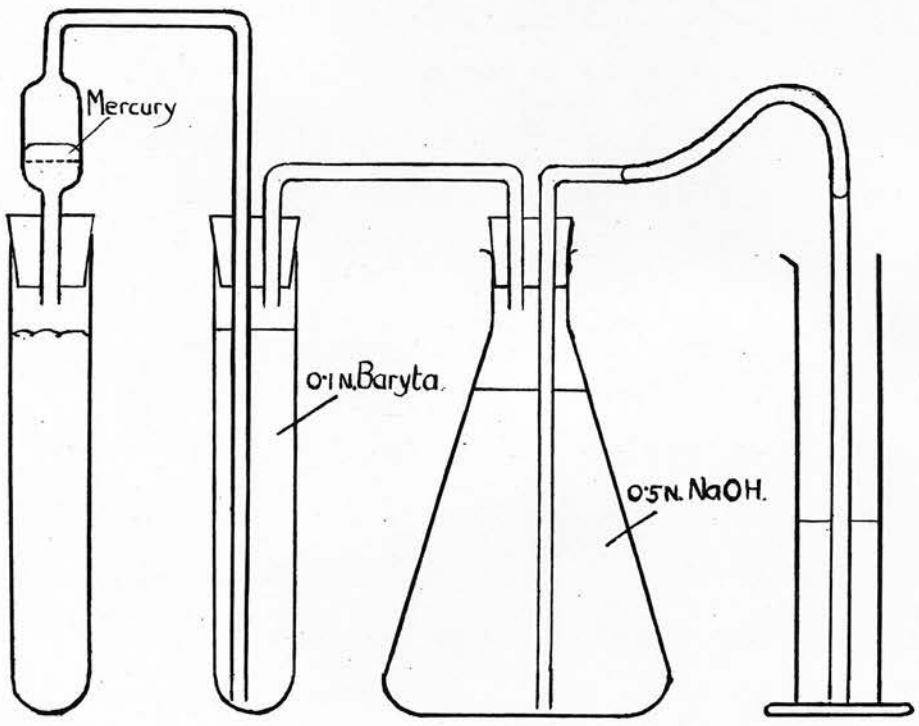


Fig. 11. Apparatus for approximate analysis of silage gases.

useful for routine analysis of farm silage.

The extract for analysis was in all cases prepared by macerating the grass or silage sample with approximately five times its weight of water in a Nelco homogeniser. The homogenate was strained through muslin and filtered through paper.

The silage gases

In most cases a rough analysis of the gases, evolved during the incubation period, was made by attaching the apparatus illustrated in Fig.11 to the silo tube and mercury valve as shown. Carbon dioxide was trapped in the tube of baryta and was estimated by back-titration with standard hydrochloric acid using phenolphthalein as indicator. The residual gas passed over into a conical flask, which initially was filled with normal caustic soda solution, and displaced its own volume of liquid into the measuring cylinder. Readings could be made at atmospheric pressure by raising or lowering the cylinder. When collection of gas was in progress, the pressure in the system was maintained slightly below atmospheric pressure by suitably positioning the cylinder. The results obtained by this method are only of value for making comparisons. Apart from inaccuracies which might arise due to the/

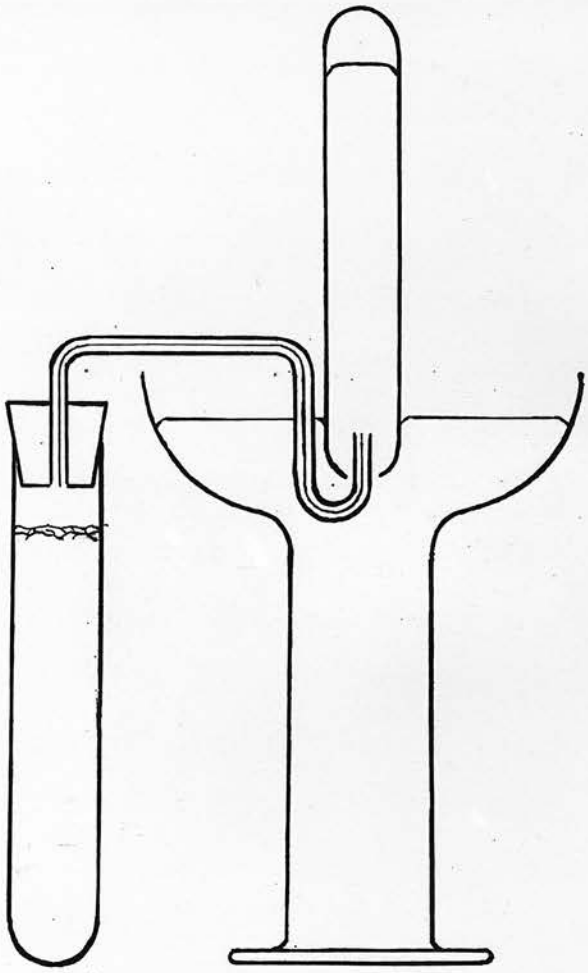


Fig.12. Collection of silage gases over mercury

the solubility of gases in the liquid displaced, a more serious error arises owing to the fact that the production of carbon dioxide in the silage tube will initially displace an equal volume of nitrogen which will be measured as residual gas.

When more precise information was required, the mercury valve was replaced by capillary tubing (1 mm. diameter) which led under mercury. This was contained in a vessel, made from a measuring cylinder and the neck of a bottle, which provided a good depth of mercury with economical quantities (Fig.12). The gas was collected in a ¾ in. x 6 in. test-tube, which was narrowed at the orifice to facilitate closure with the finger, and which was graduated in 5 ml. steps. After collection about 10 ml. of the gas was transferred to an Ambler, constant volume, gas-analysis apparatus when the amounts of carbon dioxide, oxygen, hydrogen, methane and nitrogen were determined by the standard procedures.

A number of gas samples from grass silage in tubes were analysed in the Ambler apparatus and it was found that in the cases examined only carbon dioxide and hydrogen were produced in the ensilage process (p. 131). On this basis the results obtained by the approximate method of analysis are referred to as carbon dioxide (baryta titration) and 'hydrogen' (the residual gas).

Dry matter

The dry matter content of the grass was estimated by drying to constant weight in an oven at 100°. This was usually completed in about 4 hr. No estimation was made on the 'silage'. Check weighings which were made on about fifty tube silos showed that the loss in dry matter during an incubation period of 1 week was negligible. The dry matter content of the 'silage' was, therefore, taken to be the same as that of the grass from which it was prepared.

Total nitrogen

This was estimated by the Kjeldahl procedure, as modified by Ashton (1936), using selenium as catalyst.

Volatile acids

The distillation methods of estimating volatile acids are only reliable when simple mixtures of acids are being analysed. Thus when the Wiegner or Dyer method is used (Smith, 1938) it is assumed that only acetic and butyric acids are present in silage. Consequently such procedures have only limited value for investigational work. The technique/

technique of partition chromatography on a silica gel column had been developed by Elsden (1946) and successfully employed for the estimation of the volatile acid content of rumen samples. It had the great advantage that the acids could be identified visually on the column, thus allowing the detection and estimation, not only of acetic and butyric acids, but also of propionic and valeric acids.

Preliminary work met with the difficulty of preparing a batch of silica gel which would give good separation of a mixture of these acids, but this was eventually accomplished by acid precipitation of a sample of water-glass supplied by Messrs. Crossfield & Son, Warrington.

In the first instance the percentage recovery of volatile acids, from simple or mixed aqueous solutions, was determined for the Wiegner distillation method and for the chromatographic method. For the Wiegner method the volume of solution indicated in Table 5 was acidified with sulphuric acid and distilled in steam. About 1 litre of distillate was collected, neutralised with 0.1 N NaOH to the phenolphthalein end point and concentrated to about 100 ml. on a hot plate. The Wiegner distillation was then carried out after acidifying this concentrated solution. This procedure/

Table 5. The recovery of acetic and butyric acids from aqueous solution by the Wiegner distillation technique

Volume for steam distillation ml.	Volatile Acids ml. 0.126 N				Recovery %	
	Original solution		Estimated			
	Acetic	Butyric	Acetic	Butyric	Acetic	Butyric
100	21.5	0	21.34	-1.0	99.2	-
* 100	7.15	7.06	6.60	7.39	92.3	104.7
40	0	6.28	- .05	6.33	-	100.9
100	2.75	2.15	2.42	lost	88.0	lost
"	2.75	2.15	2.69	2.65	96.4	123.2
"	2.75	2.15	2.85	2.13	103.5	99.1
"	2.75	2.15	3.00	2.14	108.0	99.5
"	2.75	2.15	2.85	2.14	103.5	99.5
		Mean of last five determinations			99.7±7.0	105.3±10.0

* This solution also contained approximately 6% W/V of lactic acid.

Table 6. The recovery of acetic, propionic and butyric acids from their solution in water or chloroform and from silage extract by the chromatographic method of Elsden (1946)

Solution for analysis ml.		Volatile Acids ml. 0.00995 N						Recovery %		
		Original			Estimated					
Aqueous	Chloro-form	Acetic	Prop-ionic	Butyric	Acetic	Prop-ionic	Butyric	Acetic	Prop-ionic	Butyric
	1.0	-	-	6.29	-	-	5.86	-	-	93.2
	1.0	1.010	0.680	0.768	1.152	0.597	0.711	114.1	87.8	92.6
	1.0	"	"	"	1.146	0.569	0.745	113.4	83.6	97.1
	1.0	"	"	"	1.122	0.662	0.676	110.9	97.2	88.2
10.0	1.0	17.00	0	13.08	15.00	0	10.50	88.3	-	80.2
*25.0					35.60	0	10.10	-	-	-
25.0		48.70	0	9.42	49.00	0	10.75	100.5	-	114
25.0		70.40	0	8.30	66.3	0	8.87	94.2	-	107.0
25.0		33.2	0	24.9	36.7	0	25.0	110.5	-	100.5
					Mean recovery from silage extract			101.7±6.7		107,1± 5.5
					Mean recovery from chloroform and aqueous solution			106.7± 10.7		90.3± 6.2

* Silage extract. The succeeding estimations were done on this extract to which known quantities of acetic and butyric acids had been added.

procedure allows the estimation of volatile acids in the presence of lactic acid (Smith, 1938). The results are shown in Table 5.

The recovery of volatile acids by the chromatographic method was determined by preparing simple or mixed solutions of the acids in chloroform and using 1 ml. of the solution for analysis. In addition figures were obtained for the recovery from mixed aqueous solutions of which 10 ml. was subjected to a double distillation using the technique of Friedeman (1938). The volatile acids in the final distillate were transferred to chloroform as described by Elsden (1946) and estimated on the silica gel column. The results obtained are shown in Table 6. They also include recovery figures obtained by this method from an aqueous silage extract (pH 4.1) to which known quantities of acetic and butyric acids had been added.

A direct comparison was now made between the Wiegner distillation method and the chromatographic method for the estimation of the volatile acids in a silage extract. The extract was prepared by mincing a farm, grass silage (pH 4.1) and extracting with cold water overnight (1 part silage to 5 parts of water). After filtering through paper, butyric acid was added to one part of the filtrate, acetic

acid/

Table 7. A comparison of the Wiegner distillation method and the chromatographic method for the estimation of acetic and butyric acids in a silage extract.

Method	Solution analysed	Volatile Acids ml. 0.00995 N / 25ml. solution Original Acetic	Original Butyric	Estimated Acetic	Estimated Butyric	Recovery % Acetic	Recovery % Butyric
Wiegner	Silage Extract			69.3	15.4		
Chromatographic				52.4	14.8		
Chromatographic				48.4	13.8		
Wiegner	Extract + acetic acid	98.2	12.7	104.5	9.2	106.2	72.5
Chromatographic		82.4	11.8	69.6	10.6	84.5	89.8
Chromatographic		82.4	11.8	72.9	10.7	88.5	90.7
Wiegner		57.0	54.3	58.7	52.8	103.1	97.3
Chromatographic		41.2	51.4	40.8	50.2	99.3	97.8
Chromatographic		41.2	51.4	41.3	50.5	100.2	98.5
	Mean recovery by Wiegner method					104.6±1.6	84.9±12.3
	Mean recovery by chromatographic method					93.1±6.4	94.2± 3.9

acid to a second part, and a third part remained untreated. All three solutions were analysed for volatile acids by the Wiegner method and in duplicate by the chromatographic method (Table 7).

It was concluded from these results (Tables 5 - 7) that the advantage of the chromatographic method lay mainly in the sure identification of the acids present. On the other hand the Wiegner procedure was simpler and involved fewer manipulations.

The paper by Hiscox and Berridge (1950), which reported the separation of the volatile fatty acids on the paper partition chromatogram, enabled the main disadvantage of the Wiegner technique to be overcome. The writer found that aqueous homogenates of grass silage could be spotted directly on to a paper sheet and developed by their method. A complete qualitative picture of the volatile acid content of the silage could thus be obtained. Whatman paper no. 54 was used in sheets (6 x 20 in.) and the chromatogram was developed by the descending irrigation method in glass tanks (18 x 8 x 8 in.). Plate 8 shows the positions taken up by single acids and the separation of a prepared mixture of acids into its constituents. The results obtained with a silage extract containing acetic, propionic, butyric, valeric and caproic acids is also shown.

All qualitative data on volatile acids reported in this thesis have been obtained by chromatography on paper. Quantitative data have been obtained by the Wiegner distillation procedure supported by the chromatographic method.

The application of the technique of Hiscox & Berridge (1950) to the analysis of silage extracts was later reported by Virtanen & Timonin (1951). Like these authors it has been found that, of all the non-volatile acids only lactic acid has ann appreciable R_F value on the chromatogram and cannot easily be distinguished from formic or acetic acids (Plate 8).

A satisfactory method for the quantitative estimation of the acid in each separated spot on the chromatogram would be most useful. Reid & Lederer (1951), who developed a similar method to Hiscox & Berridge, attempted to elute the ammonium salts of the acids from the spots and titrate them by the formal method. This proved unsatisfactory as did an attempt to estimate the ammonium radical. An ideal method would seem to be a modification of that due to Conway (1950, p.246) for the estimation of volatile acids by micro-diffusion.

Non-volatile organic acids

Lactic acid. As indicated in the introduction to this thesis (p. 31) it was of considerable interest to ascertain whether an increase in lactic acid could take place in 'silage' prepared in the absence of micro-organisms. The method of estimation, chosen for its sensitivity and accuracy, was that of Barker & Summerson (1941) which depends on a measurement of the purple-red colour formed by the interaction of lactic acid and p-hydroxy-diphenyl. This method has also been modified by Barnett (1951) for the determination of the lactic acid content of silage extracts. In his modification fresh silage (5 g.) was extracted with boiling water (100 ml.) filtered and diluted five-fold before applying the copper-precipitation treatment and colour development as described by Barker & Summerson.

The method of Barker & Summerson was first checked by the writer using aqueous solutions of lithium lactate. Initially the copper treatment was omitted in order to assess the accuracy of the colorimetric stage, but was later included so that an overall estimate of the precision of the method could be obtained. The optical density was measured on a Spekker absorptiometer, using the yellow/

Table 8. Absorptiometer readings obtained when solutions of lithium lactate were developed by the method of Barker & Summerson (1941).

Lactic acid μg.	Optical Density							
	Without copper treatment					With copper treatment		
	(a)	(b)	(c)	(d)	Mean	(a)	(b)	Mean
1.995	0.153	0.181	0.158	0.150	.161±.012	0.164	0.160	.162±.002
3.990	0.309	0.298	0.322	0.306	.309±.009	0.328	0.319	.323±.004
5.980	0.451	0.443	0.445	0.470	.452±.011	0.449	0.440	.445±.005
7.980	0.557	0.590	0.552	0.558	.564±.015	0.565	0.578	.571±.007
9.970	0.674	0.712	0.668	0.700	.688±.015	0.613	0.677	.645±.040
11.870	0.812	0.838	0.781	0.800	.808±.024	0.846	0.768	.807±.040

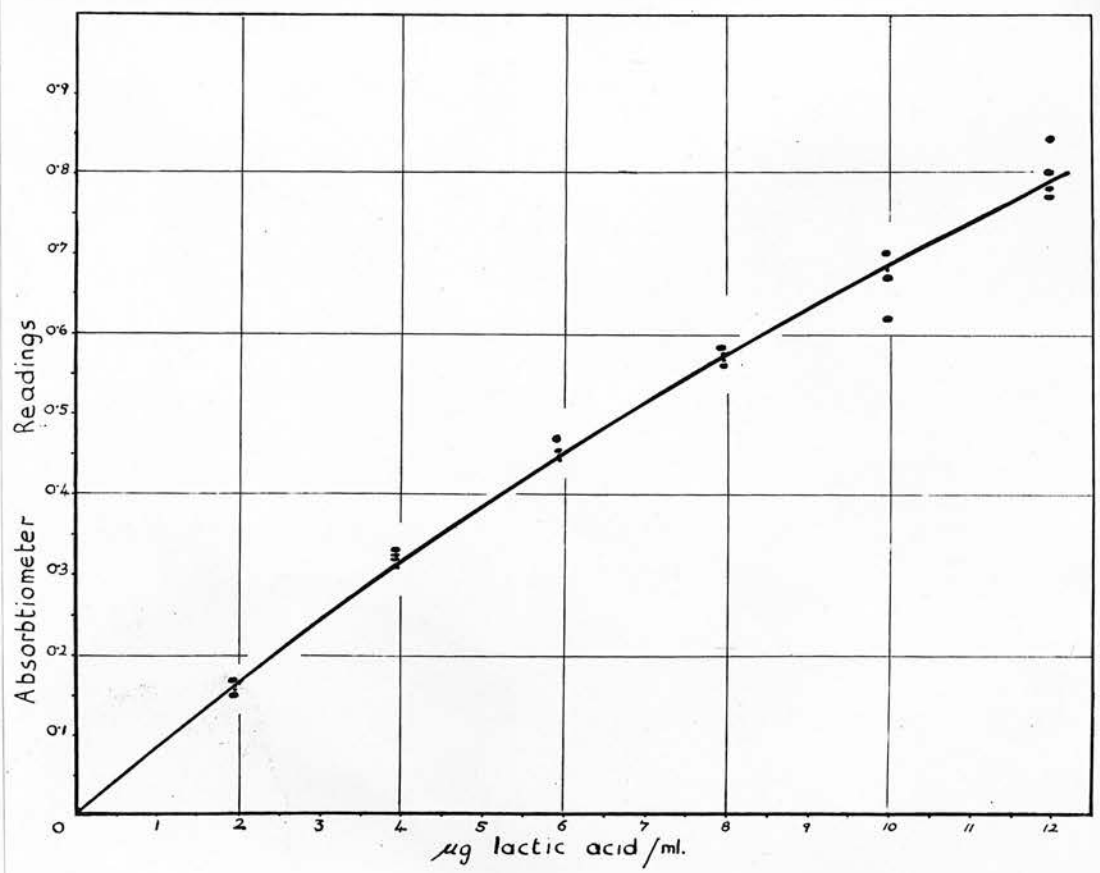


Fig13. Standard curve for estimation of lactic acid by the method of Barker and Summerson (1941).

yellow-green filter no. 605 which has its maximum transmission at 550 mμ., against a blank prepared by using distilled water instead of lithium lactate solution. Table 8 illustrates the results obtained and these have been used to construct the standard curve shown in Fig.13 - a similar curvilinear relationship was obtained by Davidson (1949) using an identical light filter. It is seen that the introduction of the copper treatment had no substantial effect on the optical density corresponding to any one lactate solution.

Since 'silage' prepared from microbe-free grass was likely to be quite different from normal grass silage it was thought preferable to check the recovery of lactic acid by this method from grass extract rather than silage extract. In any case the recovery from silage extract had been reported by Barnett (1951).

An extract was prepared by homogenizing 50 g. of perennial rye-grass, at the leafy stage, with 250 ml. distilled water and filtering. The extract was divided into two portions; known amounts of lithium lactate were added to the first and similar additions were made to the second portion after a twenty-fold dilution. In both cases 1 ml. of the solution was subjected to the copper-precipitation treatment and 1 ml. of the resultant solution was then/

Table 9. Recovery of lactic acid from diluted and undiluted aqueous extracts of perennial rye-grass using the method of Barker & Summerson (1941)

Grass Extract	Lactic Acid µg./ml. Added	Total	Optical Density (a)	(b)	Lactic Acid Recovered µg.	% Recovery
Undiluted	0	11.5	0.090	0.087	-	-
	95.00	106.5	0.560	0.560	78.5	73.7
Diluted 20x	0	0.57	-	-	-	-
	95.00	95.57	0.674	0.682	98.5	103.0
	48.70	49.27	0.376	0.379	50.0	101.5

Table 10. Recovery of lactic acid from diluted aqueous extracts of perennial rye-grass using the method of Barker & Summerson (1941)

Lactic Acid µg./ml.	Optical Density (a)	(b)	Lactic Acid estimated µg./ml.	% Recovery
0	-	-	-	-
5.25	0.035	0.034	4.5	85.7
11.08	-	0.100	12.7	114.5
25.00	0.192	0.181	23.8	95.2
33.25	0.250	0.252	32.3	97.0
51.10	0.379	0.378	50.02	98.2

then used for estimation of the lactic acid content. The aim was to obtain recovery figures under similar conditions to those employed by Barnett (1951) and also to ascertain if equally valid figures could be obtained when the extract was more concentrated. If this was so it would greatly increase the sensitivity of the method. As seen from the results given in Table 9 the recoveries obtained for the diluted extract were excellent but a very low figure was secured in the case of the undiluted extract. Davidson (1949) who has applied this method to the estimation of lactic acid in milk and milk products recorded values which were 10 - 15% too low when undiluted material was used.

The lactic acid content of 50 μg./ml. diluted extract represented about 0.6% on the fresh grass. Much smaller quantities than this were likely to occur in microbe-free 'silage' and consequently the recoveries at lower levels were investigated. Table 10 shows the results obtained with diluted extract to which smaller quantities of lactic acid (as lithium lactate) were added. It is seen that the recoveries were good at 25 μg./ml. and above but that below this level they were erratic although within an error of ±15%. It is, at any rate, quite certain that lactic acid at the level of 5 μg./ml.

of/

of diluted extract would be readily detected. This amount corresponds to approximately .06% of the fresh grass.

At the colour development stage concentrated sulphuric acid is employed. Russell (1944) found that some samples of acid failed to give full colour development and attributed this to an inhibitory action of nitrate or nitrite ions which were found in the samples in question. A similar difficulty has been encountered in this laboratory. The use of some batches of sulphuric acid of analytical reagent quality resulted in no colour development with solutions containing less than 4 μg. lactic acid /ml. but no nitrate could be detected in these samples by the ferrous sulphate test. The standard curve (Fig.13) was obtained with a satisfactory batch of acid which was employed in all the estimations of lactic acid reported in this thesis.

Non-volatile acids other than lactic acid. Since the buffer capacity of fresh grass is in part due to its organic acid content it was of interest to ascertain what changes, if any, took place in these acids during the ensilage process.

In addition to the adequate quantitative methods of Pucher et al. (1934, 1941) for malic, citric/

citric and oxalic acids, Lugg & Overell (1948) and Brown (1951) have developed qualitative methods using the paper partition chromatogram. In the method of Lugg & Overell a spot of a mixture of free organic acids is developed with a solvent such as butanol. To prevent 'tailing' of the separated spots the ionisation of the acids is suppressed by adding acetic or formic acid to the solvent. The removal of this volatile acid from the completed chromatogram, before spraying with indicator, may take several days and the method becomes tedious. The method of Brown (1951) avoids this difficulty by developing the ammonium salts of the acids. A mixture of ethanol, ammonia solution and water is used as solvent and has the virtue that the efficiency with which a mixture of acids is separated into its constituents can be adjusted by altering the quantity of water in the solvent.

Using a similar technique to that described for the analysis of volatile acids the writer has obtained the best separations with a solvent made up of ethanol (45 parts), ammonia solution 15N (2.5 parts) and water (7.5 parts). The chromatograms were run for 24 - 40 hr. at 18°C. Plate 9A shows the separation of seven non-volatile acids from a mixture of their aqueous solutions. Lactic acid can only be successfully detected with the shorter period/

period of development as it is eluted from the paper when longer periods are employed. ~~(Plate 9B)~~. Malonic, ketoglutaric and glutaric acids are not easy to identify on the chromatogram as they take up positions similar to tartaric, succinic and fumaric acids respectively. Oxalacetic and pyruvic acids failed to give any spot when developed under the conditions described above but otherwise the method was capable of detecting about 5μg. of each acid mentioned.

The technique adopted for qualitative examination of grass and silage has been to acidify the filtered homogenate with sulphuric acid to a pH of about 1; the filtrate was then extracted with ether for 48 hr. in a liquid-liquid extractor and finally a small quantity of water, usually 1 ml., was added to the extract before removing the ether by distillation. The aqueous solution of the free acids could then be spotted on the paper for development.

It would be an important addition to the technique if a method could be found for a quantitative determination of the amount of acid in each separated spot. Lugg & Overell (1948) carried out preliminary work with this aim in view but reported low recoveries when the spots were extracted with water and titrated with alkali. It was suggested/

Table 11. Recovery of lactic acid (as lithium lactate) from Whatman No. 54 filter paper by different extraction methods

Spot no.	Method of extraction	Optical Density	Lactic Acid µg./5 ml. extract	Recovery %
1	With water at room temperature	0.509	35.0	90.7±9.2
2.	"	0.444	30.0	
3	"	0.450	32.5	
4	"	0.400	26.5	
5	With water at reflux	0.455	30.5	89.2
6	None. Spot directly into water	0.500	34.2	
Paper blank	With water at room temperature	0		
Water blank		0		

suggested that the low recoveries were due to an esterification of the butanol solvent by the acid concerned. This phenomenon should be less serious using the alkaline solvent of Brown (1951). The writer has in particular investigated the recovery of lactic acid from the paper chromatogram developed by Brown's method. Two methods of estimation appeared to be worthy of trial; colorimetric estimation by the method of Barker & Summerson (1941) and a direct titration with alkali.

(1) Colorimetric estimation.

The efficiency of various extraction methods was first investigated.

Five spots (0.01 ml. ≡ 39.9 μg. lactic acid) of lithium lactate solution were spotted singly on Whatman no. 54 filter paper from a micrometer syringe and allowed to air-dry. Four spots were cut out and each extracted with 5 ml. water by frequent shaking over a period of 30 min. A fifth spot was suspended from the lower coil of a reflux condenser and extracted at reflux, using 5 ml. of water, by the method of Flood, Hirst & Jones (1948). In addition a 0.01 ml. spot of the lithium lactate solution was made directly into 5 ml. water and a blank was prepared by extracting a circle of untreated paper. Aliquots (1 ml.) of the extracts were then developed colorimetrically, omitting the copper/

Table 12. Comparison of water and 0.01 N NaOH as extractants for lactic acid (as lithium lactate) on circles of Whatman No. 54 filter paper

Spot no.	Method of extraction	Optical Density	Lactic Acid µg./5 ml.	Mean Recovery %
1	None. Spot made direct into 5 ml. water	0.410	-	-
2	"	0.250	3.20	
3	"	0.240	3.10	
4	"	0.259	3.35	
5	With 5 ml. water	0.237	3.05	98.5±4.0
6	"	0.257	3.30	
7	"	0.238	3.05	
8	"	0.257	3.30	
9	With 5 ml. 0.01N NaOH	0.248	3.15	104.9±7.9
10	"	0.252	3.22	
11	"	0.261	3.35	
12	"	0.295	3.80	
Paper blank	With 5 ml. water	0	-	-

Table 13. Recovery of lactic acid from Whatman No. 54 filter paper, in the presence of chlorophenol red indicator, by extraction with water

Spot no.	Description of Estimation	Optical Density*	Lactic Acid μg./5 ml. extract	Mean Recovery %
1	0.01 ml. Li. lactate estimated directly	0.266	3.42	
2	"	0.267	3.42	
3	"	0.270	3.49	
4	"	0.269	3.49	
5	0.01 ml. Li. lactate on paper	0.262	3.35	
6	"	0.217	2.80	89.2±5.6
7	"	0.240	3.08	
8	"	0.242	3.10	
9	0.01 ml. Li. lactate on paper sprayed with chlorophenol red	0.284	3.65	
10	"	0.307	2.97	98.5±8.2
11	"	0.282	3.65	
12	"	0.262	3.35	
Paper blank		0.080	1.0	
Paper and indicator blank		0.055	0.7	
Water blank		0		

* Spots 1 - 4 measured against water blank
5 - 8 " " paper blank
9 -12 " " paper and indicator blank

copper-precipitation treatment (Table 11).

It was concluded that extraction with cold water was as good as extraction by reflux but that the recoveries were erratic.

Extraction with cold water was now compared with extraction using dilute sodium hydroxide (0.01 N). As seen from Table 12 the recovery of lactic acid was good by both methods but occasional high values were disconcerting; they may have been caused by handling the circles of paper when transferring them to the extraction tube. In all the subsequent work recorded below, rubber gloves were worn when transferring the circles and water was used as the extraction solvent.

The effect, on the recovery of lactic acid from the spots, of spraying the dried spots with chlorophenol red indicator as in the method of Brown (1951) was now investigated. It is seen that the recovery from unsprayed paper was consistently low and from sprayed paper consistently higher (Table 13).

A critical experiment was now performed to determine the recovery of lactic acid from spots on Whatman no.54 filter paper after development with ethanol-ammonia-water and spraying with indicator. Four 0.01 ml. spots were placed in line near the top of the paper and four similar spots were made in line some distance below. Development was stopped after

3/

Table 14. Estimation of lactic acid (about 40 g.) in a spot on Whatman No. 54 filter paper by extraction with water and titration of extract

Experimental Details	Number of estimations	ml. 0.00472 N baryta Mean titre	Recovery lactic acid %
a) 3 ml. water (blank)	3	0.018	-
b) 0.01 ml. lactic acid solution titrated directly.	3	0.069±0.002	
c) 1 circle filter paper extracted (paper blank)	4	0.022±0.002	
d) 0.01 ml. spot lactic acid on paper. Extracted without drying.	4	0.065±0.004	84.3
e) 0.01 ml. spot lactic acid on paper. Air-dried before extraction.	1	0.061	76.4
f) 0.01 ml. spot lactic acid on paper, air-dried and saturated with alcohol-NH_3 solvent before extraction.	1	0.053	60.8
g) As f) but dried at 100° for 5 min. before extraction.	3	0.046±0.003	47.0
h) As f) but allowed solvent to be in contact with spot for 3 hr. before drying.	1	0.032	21.6

3 hr. when the solvent front had not quite reached the lower spots. After drying and spraying with indicator the spots were extracted with water and the extract estimated for lactic acid content as before. The lower four spots gave an average recovery of 97.1 ± 2.5% in four determinations. Extracts of the spots which had been developed with solvent failed to give any colour at all.

It was concluded that some reaction, probably an esterification, was taking place between the lactic acid spot and the alcohol solvent. Unless this could be prevented there seemed to be little hope that quantitative methods of estimating lactic acid, by a paper chromatographic technique, could be devised.

(2) Estimation by titration.

The results obtained by this method will not be reported in detail for the estimations were no more successful than those obtained by the colorimetric method. A summary of the data obtained in many experiments is presented in Table 14. It was again concluded that some reaction was taking place between the lactic acid and the solvent used for development.

The method of titration used contained some novel features. It successfully overcame the difficulty/

difficulty of titrating with very dilute alkali solutions to the phenolphthalein end-point. The circles of filter paper were extracted with 3 ml. cold water in test-tubes which fitted an EEL photo-electric colorimeter having a yellow filter with a transmission maximum at 570 mμ. After the addition of phenolphthalein indicator the tube was inserted in the instrument which could be mechanically raised until the tip of a Conway horizontal micro-burette was immersed in the liquid to be titrated (Plate 10). Baryta (0.0047 N) was then run in, while CO_2-free air was bubbled through the extract, until the end-point, which was arbitrarily selected at the fifth division of the scale, was reached. The error of titration, for a titre of about 0.2 ml., was of the order of ±0.5%.

A device using a photoelectric cell for determining the end-point of a titration has recently been described by James & Martin (1952).

Soluble nitrogen compounds: peptides, amino-acids and amines

Quantitative estimation. The colorimetric estimation of Moore & Stein (1948) had been satisfactorily tested in this laboratory for the estimation of individual/

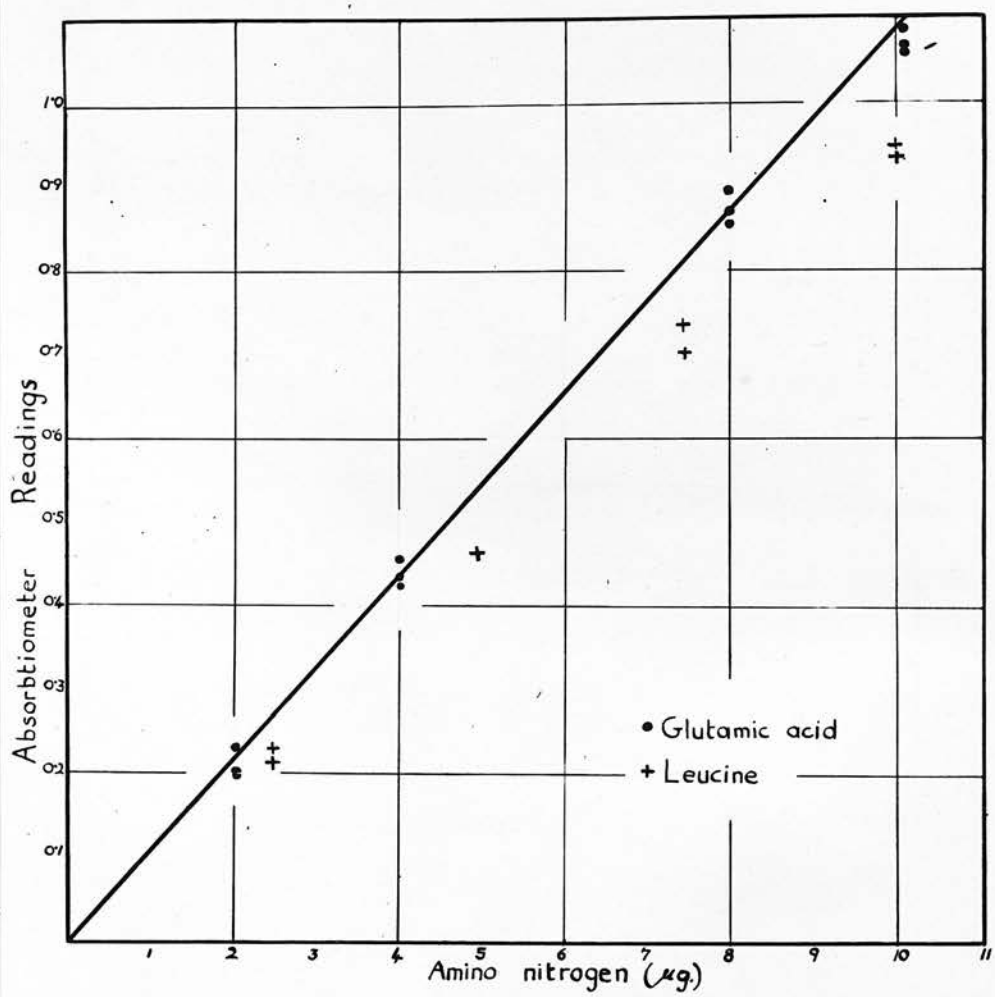


Fig15. Standard curve for estimation of amino nitrogen by the method of Moore and Stein (1948).

individual amino-acids obtained by the separation of mixtures on two-dimensional paper chromatograms and it was found convenient to employ this method, because of its sensitivity, for the estimation of the protein breakdown in the microbe-free 'silage'.

As pointed out by several workers (e.g. Smith & Agiza, 1951) there are certain disadvantages inherent in the method when applied to mixtures of substances containing NH_2-N; the colour reaction is not specific for α-amino-acids but also occurs with ammonium salts, peptides and amines, and, in addition, the various compounds do not all yield the same amount of colour per mole. Nevertheless the colour yields/mole are sufficiently similar, except for proline, hydroxyproline and cystein (Harding & MacØlean, 1916; Moore & Stein, 1948), to make comparative figures of value.

A standard curve was prepared from the results obtained from triplicate estimations of standard glutamic acid solutions (Fig.15). The values obtained using solutions of leucine are also included. Measurements were made on a Spekker absorptiometer using a yellow filter (maximum transmission at 580 mμ.). For estimation, of peptides, amino-acids and amines, in silage an appropriate aliquot of the alcoholic extract (Smith, 1938) was employed. This solution could be retained in/

in the refrigerator without deterioration and the estimation carried out on a suitable aliquot when convenient.

Qualitative estimation. It was considered important to ascertain which amino-acids were likely to be present in the early stages of the ensilage process for they might have some influence on the composition of the micro-flora. Accordingly, part of the alcoholic extract was, in some cases, concentrated in vacuo and a spot of the concentrated extract was developed on the two-dimensional chromatogram by the method of Smith & Agiza (1951) to obtain a qualitative picture of the individual amino-acids present.

Volatile bases

To allow the estimation of volatile bases to be conducted on small quantities of grass or silage extracts a micro-diffusion method using the standard Conway unit has been employed. A similar technique has been utilised for the estimation of volatile amines in biological materials including enzymic mixtures (Richter, 1937) and fish juices (Beatty & Gibbons, 1937).

The following procedure has been adopted by the writer/

Table 15. Recovery of volatile base after micro-diffusion from aqueous-solutions (1 ml.) of NH_4Cl, ethylamine and their mixture.

Diffusion Time hr.	Back titre of acid ml. 0.0107 N						Mean quantity of base diffused ml. 0.0107 N		
	NH_4Cl (a)	NH_4Cl (b)	Ethylamine (a)	Ethylamine (b)	Mixture (a)	Mixture (b)	NH_4Cl	Ethyl-amine	Mixture
1	0.319	0.322	0.390	0.405	0.242	0.230	0.280	0.203	0.364
2			0.310	0.315	0.187	0.179	-	0.288	0.417
3	0.159	-	0.182	-	0.144	0.140	0.441	0.355	0.458
4			0.200	-	0.131	0.125		0.400	0.471
5	0.082	0.078	0.155	0.140			0.520	0.453	
6			0.145	0.143	0.100	-		0.456	0.500
7									
8	0.084	0.076					0.520		

By distillation into standard acid 1 ml. NH_4Cl solution ≡ 0.542 ml. 0.0107 N base.

" " " " " 1 ml. ethyl-amine solution ≡ 0.443 ml. 0.0107 N base.

" " " " " 1 ml. mixed solution ≡ 0.492 ml. 0.0107 N base.

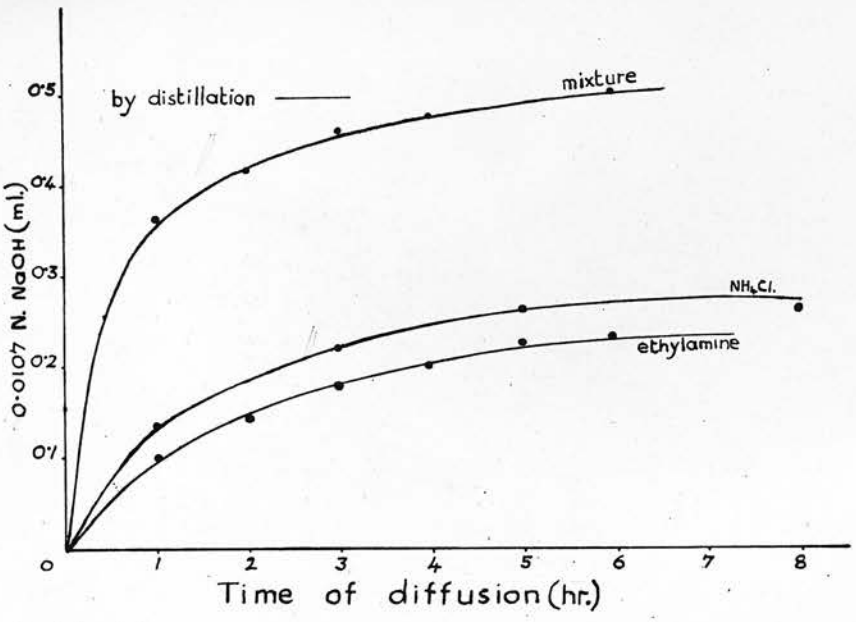


Fig.16. Rate of diffusion of volatile bases from aqueous solution in a standard Conway unit at room temperature.

writer. Into the centre compartment of the unit is pipetted 1 ml. $^{N}/_{150}$ HCl solution containing methyl red-methylene blue indicator and 1 ml. of the filtered grass or silage homogenate is transferred to the outer compartment. To the latter approximately 1 ml. saturated K_2CO_3 solution is then quickly added and the unit is immediately sealed. Diffusion is allowed to continue at room temperature overnight before back-titrating the acid with 0.01 N NaOH using a micro-burette.

Experiments were carried out to ascertain the recovery of bases, by this method, from aqueous solutions of NH_4Cl, ethylamine, and their mixture (1 part to 1 part). The result obtained after various periods of diffusion are shown in Table 15. Satisfactory agreement between duplicate determinations was obtained in the later periods when the diffusions were virtually complete (Fig.16). The graphs in Fig.16 have been constructed from the data in Table 15, the values plotted for NH_4Cl and ethylamine having been calculated on the basis of 0.5 ml. of each solution. It is seen that the results obtained by diffusion are very close to those obtained by a distillation, under alkaline conditions, into standard acid.

A further comparison was made between the micro-diffusion and distillation method by determining/

Table 16. Comparison of micro-diffusion and distillation methods for for estimating volatile base in silage extracts.

Solution used	Volatile base / ml. solution ml. 0.00995 N					
	By micro-diffusion	Calculated	% Recovery of added base	By distil-lation	Calculated	% Recovery of added base
Dilute silage extract A	0.470	-	-	0.450	-	-
NH_4Cl	0.58, 0.57	-	-	0.630	-	-
Diluted extract A + NH_4Cl(3)	0.50, 0.50	0.522	92.4	0.570	0.540	109.5
Diluted extract A + NH_4Cl(4)	0.55, 0.55	0.564	99.1	0.610	0.594	103.2
Silage extract B	0.355 0.365	-	-	0.354		
Silage extract C	0.450 0.458	-	-	-	-	-
Silage extract D	0.475 0.473	-	-	0.502		
Silage extract E	0.560 0.564	-	-	0.572		

determining the volatile base present in a farm silage extract to which different amounts of an NH_4Cl solution had been added. The extract was made by homogenizing 50 g. silage with 250 ml. water. Preliminary experiments showed that the volatile base content was high and a five-fold dilution was made to bring the content of volatile base within a suitable range. The following solutions (1 ml.) were then analysed by the micro-diffusion method:

(1) the diluted extract
(2) NH_4Cl solution
(3) 5 ml. diluted extract + 5 ml. NH_4Cl solution.
(4) 2 ml. " " + 8 ml. " "

Alcoholic solutions were also prepared by diluting 60 ml. of each of the above solutions to a final volume of 200 ml. with 90% neutral alcohol (Smith, 1938), and the volatile base in the alcoholic solution was estimated by the distillation procedure. It is seen from Table 16 that both methods were somewhat erratic but the micro-diffusion method was, if anything, more accurate than the distillation procedure. In Table 16 are also recorded the results obtained by the two methods with aqueous and alcoholic extracts from grass silage prepared in test-tubes and incubated for 22, 33, 51 hr. and 8 days (extracts B, C, D, E). The agreement/

agreement was satisfactory. In all cases the micro-diffusion method gave a lower result than the distillation method, possibly due to the continuous but slow breakdown of nitrogen compounds which is known to take place at the distillation temperature.

Considerable experience with the micro-diffusion method has since shown that it is capable of providing accurate results if aqueous silage extracts are used. Alcoholic solutions, however, cannot be examined by this method presumably owing to interference by the alcohol which also diffuses over into the central compartment of the unit.

Alcohols

The majority of the methods for determining the amount of ~~alcohol~~ ethanol in biological material depend on the oxidation of the alcohol to acetic acid by a dichromate-sulphuric acid mixture followed by an estimation of the quantity of dichromate which has been reduced. Such methods really estimate the total amount of volatile substances capable of reducing dichromate, including alcohols and aldehydes. For convenience, such substances are referred to as alcohol in the remainder of this thesis.

Before estimation, the alcohol is separated from the material by distillation, aeration or diffusion/

Table 17. Recovery of alcohol from silage extract by the micro-diffusion method of Winnick (1942).

Solution analysed	ml. 0.1 N sodium thiosulphate						
	Alcohol added / 1 ml.	Back titre (a)	(b)	Mean	Alcohol estimated Total	Added	Alcohol recovery %
Water	0	1.880	1.915	1.898			
Aqueous alcohol (A)	1.381*	0.53	0.55	0.54	1.358	1.358	98.4
Silage extract (S)	0	1.565	lost	1.565	0.333	-	-
* S_8A_1	0.151	1.458	1.462	1.460	0.438	0.142	94.2±1.4
S_2A_1	0.4527	1.249	1.234	1.242	0.656	0.434	95.0±1.6
S_1A_1	0.679	1.062	1.062	1.062	0.836	0.670	98.6±0

* Estimated by a determination of specific gravity of absolute alcohol followed by a five-hundredfold dilution.

* Subscript numbers indicate ratio in which solutions were mixed e.g. S_1A_1 ≡ 1 ~~part~~ volume silage extract + 1 ~~part~~ volume aqueous alcohol.

Table 18. Recovery of alcohol from grass and silage extracts and homogenates by the method of Ryan, Nolan & Conway (1948).

Solution analysed	Alcohol added	ml. 0.1 N sodium thiosulphate Back titre (a)	(b)	(c)	Mean	Alcohol estimated Total	Added	Mean alcohol recovery %
Water	0	0.300	0.300	0.300	0.300			
Aqueous alcohol (A)	0.1383	0.163	0.167		0.165	0.135		97.6
Grass extract (G)	0	0.288	0.290		0.289	0.011		
G_2A_1	0.045	0.249	0.246		0.2475	0.0525	0.0452	100.4±3.3
G_1A_2	0.090	0.206	0.204	0.208	0.2060	0.0940	0.0903	100.3±1.8
Silage extract diluted 2x ($\frac{1}{2}$S)		0.183	0.182	0.182	0.1823	0.1177		
S_1A_1	0.0675	0.114	0.114	lost	0.114	0.186	0.068	100.8±0
S_1A_2	0.0900	0.132	0.135	0.131	0.133	0.167	0.0885	98.3±1.5
Water	0	0.298	0.298	0.298				
Aqueous alcohol (A)	unknown	0.154	0.155	0.155	0.155	0.143		
Grass homogenate (G)	0	0.252	0.251	0.250	0.251	0.047		
G_2A_1	0.0477	0.218	0.221,	0.222	0.220	0.078	0.0467	97.7±3.6
G_1A_2	0.0953	0.188	0.192	0.189	0.190	0.108	0.0923	96.8±1.9
Silage homogenate (S)	0	0.101	0.089	0.090	0.095	0.203		
S_1A_1	0.0715	0.129	0.129	0.124	0.127	0.171	0.0695	97.2±1.9
S_1A_2	0.0953	0.142	0.140	0.141	0.141	0.157	0.0893	93.7±0.9

diffusion. Diffusion methods have been based on the original method of Widmark (1922) and more recently Winnick (1942) described a modification of this method, for use with blood or urine, in which a standard Conway micro-diffusion unit was employed.

In this laboratory Winnick's method has been employed for the estimation of alcohol in silage extracts using 1 ml. quantities. The results given in Table 17 indicate the accuracy of the method and the satisfactory recovery of alcohol when known amounts are added to silage extracts. Smaller quantities (0.1 ml.) may be employed without reducing the sensitivity of the method if the modification of Ryan, Nolan & Conway (1948) is used. Details of this modification were given by Conway (1950, p.251). Its application to the analysis of grass and silage extracts has given very satisfactory results (Table 18). Slightly lower recoveries of alcohol were obtained from those mixtures prepared by adding known quantities of alcohol to unfiltered homogenates which were then filtered before analysis.

The mean back-titre of 0.01 N $Na_2S_2O_3$ in five determinations of the alcohol content of a grass extract was 0.290±0.0015 ml. equivalent to an alcohol content of 0.033±0.005% on a dry matter basis. The accuracy of the method at low alcohol concentrations/

concentrations may be considerably increased by using 0.5 ml. (instead of 0.1 ml.) of extract for the determination (Conway, 1950, p.252). Thus a similar set of determinations on a different grass extract using 0.5 ml. gave an alcohol content of 0.028± 0.0004% on the dry matter.

DETAILS OF ATTEMPTS TO GROW MICROBE-FREE TIMOTHY AND TO PREPARE STERILE 'SILAGE'.

Experiment 6*

Microbe-free timothy seed was sown on August 15th, 1950, in the sterile chamber, by the method previously described (p.37), and covered with a layer of sand which had been placed for this purpose in one of the large crystallising dishes. No great difficulty was experienced in these operations. The sand, however, had become damp during the sterilisation process and was inclined, when touched, to adhere to the inner surface of the gloves.

On the second day after sowing, both layers of cellophane split at two corners; the material had shrunk after sterilisation on to the metal corners of the upper framework which had pierced the fabric. The holes were immediately patched with sterile cotton-wool and cellophane in the hope that the ingress of micro-organisms had been prevented by the out-flowing air stream, but mould colonies developed on August 25th on the sand immediately below the corners concerned. The experiment was, therefore, abandoned.

Another fault was also noted during this period; the gloves had been used three times for watering the/

* for Exp. 1-5 see p.39-44

the dishes and on the third occasion the rubber was observed to have perished at the junction between gloves and chamber. Presumably this was due to sterilising the glove cuffs in a slightly stretched condition but the fact that they had been bound on to the projecting metal rims with an adhesive cellulose tape may also have been a contributory factor.

Experiment 7

Modifications to growth-chamber. The largest gloves available (size 9½) were used but these were still stretched when fitted to the metal rims. They were bound in position with cotton-wool and string. In order to reduce the strain on the rubber to a minimum, the gloves were not used for watering the plants. Instead, the outer dishes were omitted from the chamber and the inner ones, having a hole in each base, were placed on a shallow, enamel tray which just fitted into the base-part. The tray could be flooded with water from the reservoir by siphoning and thus provided sub-irrigation for the plants. As an additional precaution the gloves were protected from sunlight by covering with thick paper.

It had been found in Exp. 6 that the inner cotton-wool joint between the base-part and the upper/

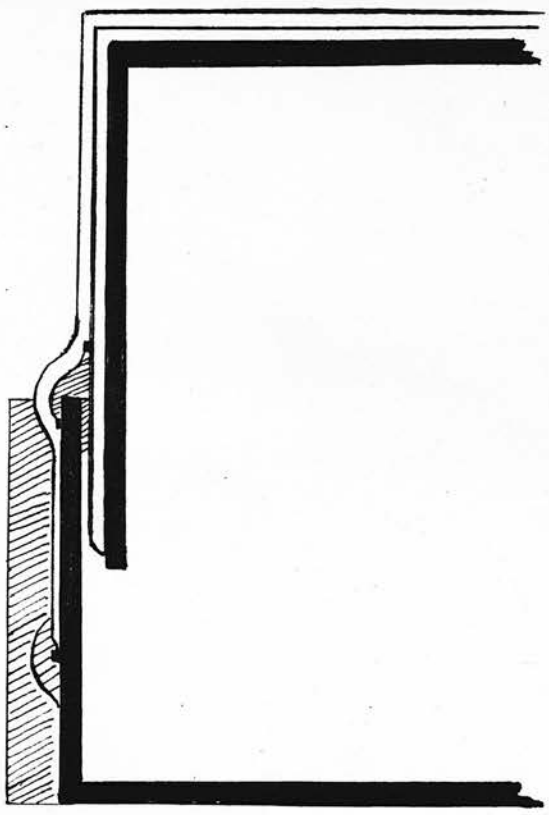


Fig. 17. Showing position of additional cotton-wool joint used in exp. 7 onwards.

upper framework (Fig. 9, p.49) was not easily made efficient owing to the uneven surface of the metal strips. In order to improve the joint another length of cotton-wool was stuck to the inner layer of cellophane and to the top edge of the base-part before the second sheet of cellophane was fitted (Fig. 17).

Air supply. It was noticed in Exp. 6, and again in this experiment, that none of the air pumped into the chamber came out of the exit filter although a slight positive pressure in the cabinet was indicated by a partial inflation of the rubber gloves when the pump was started. It was presumed that the air was escaping through the cotton-wool joints and then via the folded ends of the cellophane sheets (see Exp. 12). These were stuck down with cellulose tape but it was impossible to obtain a gas-tight seal in this way since the adhesive tended to deteriorate at a cellophane-cellophane joint during sterilisation.

Growth and harvesting of plants. Microbe-free timothy seed was sown as before on Sept. 8th, 1950, and the chamber was placed in the greenhouse. Germination took place on Sept. 15th. The weather was dull and stormy throughout the growth period and the use of an electric fire was needed to maintain a reasonable temperature. The dishes were watered every/

every two days, the amount of water run on to the tray being adjusted so that it was just completely absorbed by the sand.

After 4 weeks growth the grass was about 4in. high and was beginning to show signs of bleaching at the tip of the blade. The cause of this was not known. The crop was cut and part of it packed into the silo tube on Nov. 3rd, 1950 when it was about 5in. in height. At this time some of it was leaning and touching the cellophane near the top of the upper framework. Tip-burn was still evident but not serious and otherwise the crop looked healthy. The leaf blades were very narrow and tender.

Bacteriological tests on the grass and silage. As previously described (p.38) samples of the grass leaf and of the sand medium were tested for sterility by dropping them into broth before the chamber was opened. In all cases a pure culture of Penicillium grew in the broth. No bacteria could be found when it was streaked on to nutrient agar and incubated at 22°.

Microscopic examination of the grass and of the resulting 'silage' revealed no fungal hyphae and it was concluded that the fungus was present mainly as spores.

A portion of the silage (4g.) was homogenized in/

in a sterile tube with sterile water (30 ml.) and 1 ml. quantities of the homogenate were plated in duplicate on tomato agar and nutrient agar. After 7 days incubation at 22° only one colony - a micrococcus - appeared on one of the nutrient agar plates. No organisms could be found in a Breed smear prepared from the homogenate. It was concluded that no microbial growth had taken place in the 'silage' tube and that the fungal spores on the grass were present in low numbers.

Cause of contamination. It was of great importance to ascertain the origin of these spores. The inlet filter was examined, after the experiment was completed, by cracking it at various levels and removing samples of the cotton-wool aseptically. These were washed in a little Ringer's solution and the wash plated on nutrient agar. No growth was obtained from samples taken below 10 cm. from the top of the filter which, therefore, appeared to have worked efficiently. The seed which had been used in the experiment was therefore, suspect but no additional check could be made on this.

This suspicion was supported by the following experiment. The sterilised chamber, containing only swabs and tubes of broth, was re-assembled. The air-stream after passing the filter was allowed to bubble through one of the tubes of broth which was changed/

changed weekly using the gloves. At the same time swabs were taken of the sides and bottom of the base-part and dropped into separate tubes of broth. After 4 weeks no visible growth could be detected in any of the broth tubes and no growth occurred when the broth was streaked on nutrient agar and incubated at 22°.

Experiment 8

Modifications to growth chamber. Since the exit filter had been found to be redundant it was not fitted in this experiment. Instead, the metal outlet tube was bunged with rubber and a cotton plug, and then securely wrapped with cotton-wool and cellophane.

In order to utilise the interior of the chamber more efficiently the crystallising dishes were replaced by two domestic pyrex dishes (26 x 16 cm.) each having two ¼in. diameter holes drilled in the bottom and each occupying about half of the enamel tray.

Growth and harvesting of the plants. Sowing took place on March 20th, 1951, in very cold weather; the seed was not covered with sand. Until germination occurred on March 31st the chamber was kept in the laboratory, the illumination being supplemented during/

during the day by light from a 400w. high-pressure, mercury lamp at a target distance of 3ft. Subsequently the chamber was removed to the greenhouse and only natural illumination was employed. The germination was very uneven; in a half of one dish on April 17th the grass was 2in. high and growing thickly whereas in the remaining half practically no seedlings were present. No explanation for this can be advanced. The dishes were not, however, watered until germination was almost complete and the possibility exists that some inhibitory concentration of salts had accumulated at the surface of the sand during sterilisation.

Growth was very slow during May due to cold, sunless weather and when conditions did improve in June symptoms of tip-burn again appeared and became serious. The information gained from Exp.10 which was running concurrently, indicated that the trouble was due to heat radiations and consequently the cabinet was shaded with cheese-cloth during periods of intense sunlight. The crop made a good recovery under these conditions and when harvested on July 7th it was about 6in. high and growing very thickly; the leaf blade was again very narrow. There was a fair quantity of dead tissue present at this time resulting from the heat damage which occurred earlier.

The silage tube was approximately two-thirds full when the half of the crop to be ensiled had

been/

been packed in.

Bacteriological tests on the grass and silage. Tests on the sand medium and the grass, carried out as before, again revealed the presence of an unidentified mould apparently present as a pure culture. No mycelia could be detected microscopically on the grass or in the resulting silage. Platings of the aqueous homogenate of the silage or glucose agar gave a pure culture of what appeared to be the same organisms in numbers equivalent to 850/g. fresh silage. It is unfortunate that no counts were made on the fresh grass. Thus no quantitative evidence is available to prove that no microbial metabolism took place during the incubation period. Nevertheless the rapid establishment of anaerobic conditions which takes place in these tubes (p.142) would seem to preclude the possibility of extensive aerobic metabolism.

Origin of contaminant. Tests carried out on the filter in the same way as detailed in Exp.7 again failed to reveal the source of contamination.

Experiment 9

Modifications to growth-chamber. One of two identical new growth-chambers was used in this experiment. It had no outlet tube for the air (see p.97) and/

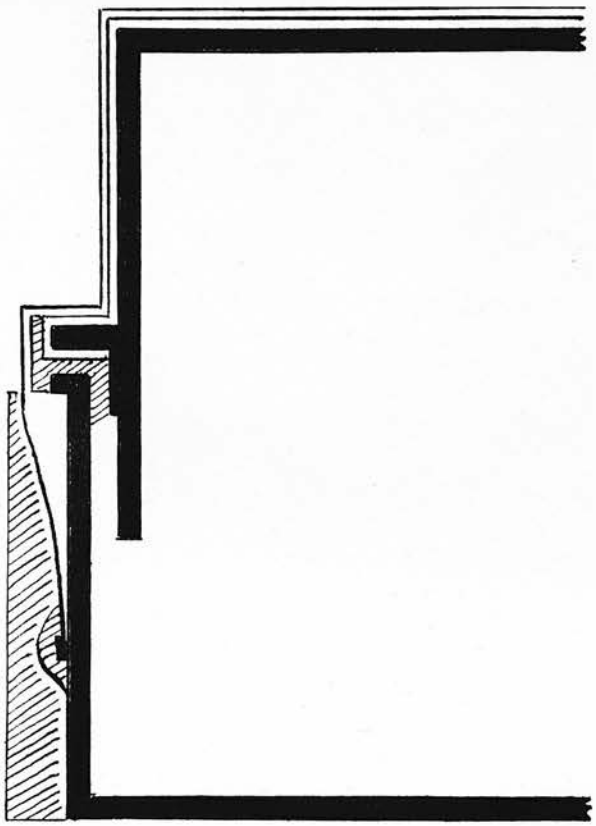


Fig. 18. Transverse section of part of growth-chamber used in Exp. 9.

and the top framework was slightly modified to allow a better joint to be made with the base-part. This was accomplished as follows: to the framework was soldered a metal projection (Fig.18) under which was stuck the edge of the first layer of cellophane; to that part of the cellophane which was under the projection two layers of neck wool were now fixed with rubber solution. Before sterilisation a 3in. wide strip of cotton-wool was placed along the top edge of the base-part and allowed to drape down the inner and outer surfaces; it was held in position with adhesive cellulose tape. Thus, when the upper framework was fitted, the thick pad of cotton under the metal projection bedded on to the upper edge of the base-part and, at the same time, a tight cotton-wool joint was formed between the two parts of the chamber. The joint was reinforced by a further wrapping of wool which was fixed with rubber solution (Fig.18) before the second layer of cellophane was fitted as previously described.

Two pyrex dishes were again used to hold the sand medium.

Growth and harvesting of the plants. Sowing took place on May 11th, 1951, and the chamber was placed in the greenhouse. In order to ascertain if conditions inside the chamber were in any way restricting growth, seed was also sown at the same time/

time in unsterile sand medium placed in a crystallising dish outside of the chamber. For some reason the germination and growth in this dish was much superior to that in the dishes in the growth-chamber. Thus on May 25th, the seed sown in the crystallising dish had germinated uniformly and the seedlings were a good 2in. high whereas in the chamber, germination had been patchy and the seedlings were lin. high at the most. Because of this poor germination in the chamber the sand was kept well-watered in order to keep the nutrient concentration as low as possible.

At the beginning of June the natural illumination was supplemented by use of a 500w. high pressure mercury lamp from 6p.m. to 12p.m. daily at a target distance of 3ft. (Plate 11). It was hoped that this would speed up the growth.

On June 28th most of the plants were still only about lin. in height and yellow in colour. It was noted that the siphon arrangement had been continuously leaking water into the tray and it is possible that the abnormal growth was due to excess water in the sand medium. At any rate improved growth occurred when the leak was checked.

A further mishap occurred on July 7th when the air-filter was accidentally broken. The crop was, therefore/

therefore, abandoned from the point of view of obtaining sterile 'silage', but it was allowed to continue growing to determine to what degree of maturity it would attain. By mid-September the crop was touching the cellophane roof. The leaves were broad and more typical of timothy than any crop yet seen in the chamber. There was, however, no signs of flower heads. It thus appeared that the conditions of growth and the height of the growth-chamber were such that a mature crop could not be obtained.

Cause of poor germination. While Exp.9 was in progress four crystallising dishes were filled with sand medium and two of them sterilised under the same conditions as used for the growth-chamber. Each dish was then sown with timothy seed, the seed in one sterile dish and in one unsterile dish being covered with sand, and placed near the growth-chamber in the greenhouse. In those dishes in which the seed was covered germination was quicker and more uniform than in the remaining dishes. There was no evidence to suggest that the sterilisation of the nutrients in the sand had any ill effect on the plant growth. The dishes used, however, had no hole in the bottom and were consequently irrigated from the top. It was still possible that in the chamber it would be advantageous to flood the surface of/

of the sand before sowing. This precaution and the covering of the seed after sowing was, therefore, indicated.

Experiment 10

The object of this experiment was to examine the possibility of growing timothy in the growth-chamber using only artificial illumination.

The design of the chamber was similar to that used in Exp.9.

Growth and harvesting of the plants. The seed was sown, without covering with sand, on May 11th, 1951, the same day as for Exp.9. The chamber was placed in an air-raid shelter whose only illumination was from three parabolic reflectors carrying a 500w. high-pressure mercury vapour lamp and as many tungsten lamps as required (~~Plate 12~~). A target distance of 3 ft. was employed.

Until May 24th only light from the mercury lamp was used in order to avoid heat injury to the young seedlings. The daily period of illumination was 5p.m. to 9a.m. When the light was switched on a temperature of between about 5° and 13° occurred, at the outer surface of the cellophane top (see Appendix 3). The germination in this experiment although slow was much superior to that of Exp.9.

On May 24th the light from four 50w. tungsten bulbs was mixed with that from the mercury lamps but by June 14th severe tip-burning had developed and the tungsten bulbs were removed. The recovery of the plants was rapid and on June 28th the plants were growing vigorously and were an intense green in colour. Tungsten lamps to the value of 160w. were inserted in the reflectors at this time but their wattage was reduced by half on July 11th when slight tip burn was again noticed. No further change in illumination was made during the growth period.

The crop was harvested on August 17th, 1951. It was very thick at the bottom and about 5-6in. tall but it compared unfavourably in height with timothy growing, under the same conditions of illumination, outside of the chamber (Plate 13). Both crops had narrow, wiry dark green leaves. Just before cutting began, a small fly was observed inside the chamber. Closer inspection showed that the inner cellophane layer had a split of some 2in. near the centre of the top of the chamber. It was concluded that the fly probably in the larval state must have penetrated the outer cellophane via one of the end folds and entered the chamber via the split inner cellophane layer.

When the silo tube had been packed with half of the crop it was completely full. The remaining crop was/

was cut into a beaker and covered before dismantling the chamber so that a microbial count could be made on the fresh grass.

Bacteriological tests on the grass and silage. After homogenising 10g. of the grass with 40 ml. sterile water in a sterile apparatus, 1 ml. of the homogenate was plated on tomato agar and incubated at 22°C for 3 days. The plate was crowded with bacterial colonies which were uncountable. Only one species of bacteria could be found on the plate by examining a number of colonies. It was identical with that found in the silage homogenate. The latter was examined in detail by plating suitable dilutions on glucose yeastrel agar (total count), ammonium lactate agar and acetate agar (Keddie, 1952). On the first two media a pure culture of Pseudomonas was obtained in numbers equivalent to 76 millions/ml. homogenate. Dilutions were also prepared in a medium containing yeast autolysate, lactate and acetate, and also in a medium containing gelatine and peptone (Rosenberger, 1952) in order to determine the numbers if any of lactate-fermenting and proteolytic anaerobes respectively. No growth was obtained in these media.

It was concluded that some development of the Pseudomonas might have occurred during the aerobic phase in the silo tube but that no other microbial activity had taken place during the incubation period.

The species of Pseudomonas isolated was strictly aerobic/

aerobic, capable of rapidly liquifying gelatin (24hr.) and actively lipolytic; lactose and glucose were fermented but no fermentation of sucrose occurred.

Experiment 11

In this experiment an attempt was made to eliminate all possible sources of contamination in the light of past experience.

In the first place the seed, which had been treated with hypochlorite as before, was more rigidly tested. It seemed possible that a few fungal spores which had escaped the sterilisation process, were not included in the relatively small sample taken for the check on sterility, or that they would not find the conditions in the broth tube suitable for germination. In addition to the tests previously described, therefore, about half of the dried seed which had been treated with hypochlorite was poured into a thin layer of sterile glucose broth contained in a 100 ml. conical flask. The amount of liquid (10 ml.) was such that most of it was absorbed by the seed and the conditions around the seed must therefore have been completely aerobic. Many of the seeds germinated on incubation at 22° for 10 days. No micro-organisms could, however, be cultivated from the flask during this incubation period nor

were/

were any visible microscopically.

Secondly an attempt was made to reduce the possibility of organisms penetrating the air-filter. A small sterile filter (4 x 1¼in.), was attached to the pump side of the inlet filter and was replaced weekly by a similar sterile filter. In this way the 'strain' on the larger filter should have been considerably reduced.

The chamber used was that of the first design (Exp.6). Again careful attention was paid to the end folds of the cellophane. These were carefully sealed with cellulose tape before sterilisation and after sterilisation a further layer of tape was used to make the joints secure.

Growth and harvesting of the plants. Before sowing, the sand surface was flushed with water. The seed was sown on October 17th, 1951 and covered with dry sand. This had been prepared by sterilising in a screw capped bottle at 2 atm. steam pressure for 1hr. followed by a dry sterilisation with the cap off at 180° until dry; the cap was then fitted tightly and the bottle placed in the growth-chamber for a further sterilisation. The growth-chamber was transferred to the greenhouse where the natural light was supplemented with that from a 500w. mercury lamp. Germination took place, evenly but thinly, around October 29th. The lamp was used for 6hr. daily until November 18th when/

when the plants were about 2in. high and the supplementary illumination was then increased to 8hr. per day.

Harvesting was carried out on Feb. 21st, 1952. There was a small amount of tip-burn and a few leaves were completely bleached. Otherwise the crop was green but the leaf blade was narrow as in most of the previous experiments. The silo tube when packed had an air space of about lin. above the grass. In place of the mercury valve the stopper used for closing the tube carried a bent capillary (Fig.12) which had a ½in. long plug of cotton-wool in the middle of the horizontal portion. The escaping gases could thus be collected over mercury. The latter was dried at 120° before use to avoid the possibility of moisture wetting the cotton plug.

Bacteriological tests on grass and silage. The sand medium and the grass when inoculated into broth showed the presence of a mould (**Penicillium**) and bacteria (Gram-positive rods). When 0.1 ml. of a homogenate of the grass was plated on glucose-yeastrel agar the same mould and bacterium were present in uncountable numbers. The latter was a corynebacterium having the following properties: glucose fermented to pH5; lactose and litmus milk not fermented; nitrate reduced; acetoin not formed; facultative anaerobe. Acetic acid was the only fermentation product which could be/

be detected by chromatography.

A count of the corynebacterium was made on the silage by plating dilutions of the homogenate on double-layered, glucose yeastrel agar plates and 19 millions/ml. homogenate were found at 30°. The fungal count on single-layered plates at 22° was 24,000 / ml. homogenate.

Origin of contaminants. The air-filter was tested by cracking it at various levels and removing plugs of cotton which were dropped aseptically into bottles containing nutrient broth. After incubating the cultures at 22° for 3 weeks no growth was obtained from samples below 10 cm. from the surface of the cotton. From the sample taken at 10 cm. depth only a yeast could be cultivated.

Experiment 12

This was a repeat of Exp.10 in which only artificial illumination was employed. The chamber used was that of the second design (Exp.9) and the additional precautions outlined in Exp.11 were again observed.

Growth of plants. The seed was sown on October 19th, 1951 and germinated about October 31st. For the first three weeks of growth illumination was by a 500w. mercury lamp for a daily period of 8hr. and this/

this was gradually increased over a 3 week period to 16hr. per day. In mid-December there was slight tip-burn visible when 30w. tungsten light was introduced. The tip-burn increased, however, and the tungsten lamps were removed in early January. By the middle of January, however, the plants in one dish were partly wilted. In February the crop in one dish had completely failed and mould growth could now be observed in the bare patches. As the crop was not worth harvesting an attempt was made to trace the source of contamination before the chamber was dismantled.

Origin of contaminant. The air-filter was removed and a blue smoke, generated by dropping a Waxoline dyestuff on to a hot plate, was passed into the chamber under pressure. After about 15 min. of this treatment a number of blue patches were visible on the outer cotton-wool of the inner joint (Fig.18) showing either that the smoke particles were getting past the inner joint or that the inner layer of cellophane was punctured. The chamber was now carefully dismantled. One blue spot was found to be due to a lin. split in the inner cellophane layer; two small spots resulted from a loose joint with adhesive cellulose tape at an end fold; and a fourth spot was caused by a loose packing of the cotton-wool of the inner joint at one corner.

Table 19. Yields of timothy from growth-chamber and weights of grass analysed and ensiled.

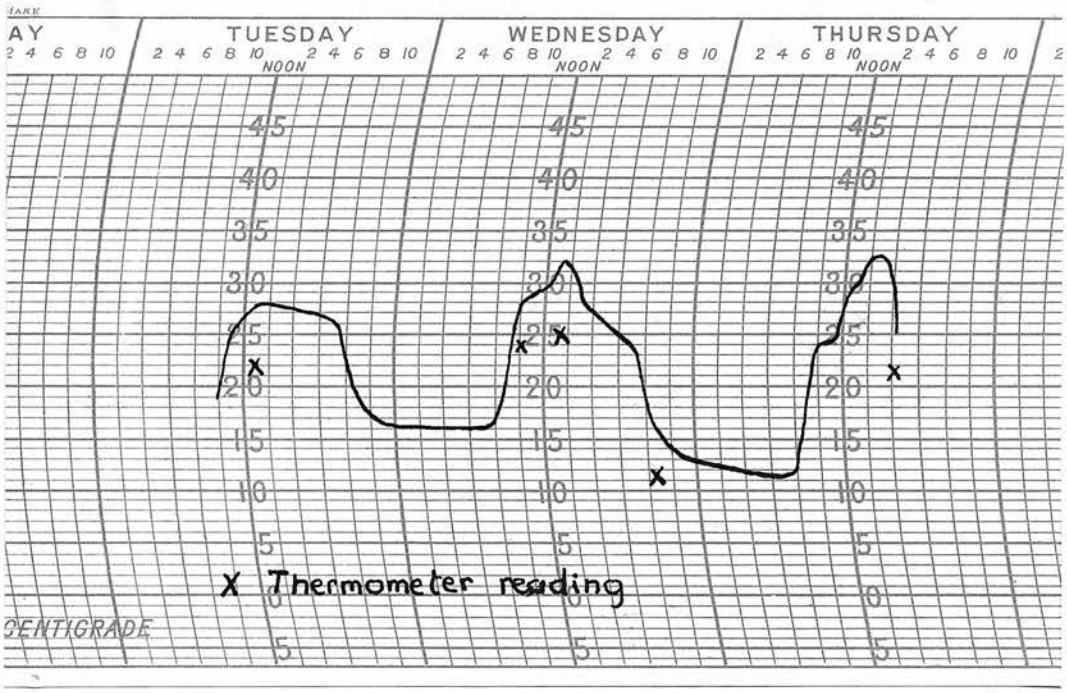
Exp. No.	Growth Period (weeks)	Illumination	Wt. Grass (gm.) used for			Total Yield (g.)
			Drying	Homogenising	Ensiling	
7	7	Daylight	0.1705	1.490	6.678	8.338
8	13	Daylight	3.025	9.119	13.048	25.192
9	16	Daylight + Artificial	2.130	5.223	15.210	22.533
10	12	Artificial	0.730	10.051	12.489	23.270
11	12	Daylight + Artificial	1.435	6.119	8.730	16.284

Yields of grass from growth-chamber

The weight of grass obtained from the growth-chamber in each experiment is shown in Table 19. It would appear that the maximum yield, which can be obtained from the chamber under the conditions which were employed, is about 25g. fresh weight. Of this about 15g. is required for ensiling in order to fill the silo-tube.

Experiment 13

It had become increasingly evident that the conditions of growth inside the chamber were not ideal. The most likely deterrent to satisfactory plant growth appeared to be over-heating. In all the experiments in which plants had been grown in the chamber the temperature recorded by a thermometer placed on the outer surface of the cellophane had been taken as a reasonable guide to the temperature inside the chamber but the prevalence of tip-burn of the grass and its association with tungsten light in these experiments where artificial illumination was employed, suggested that this assumption was unwarranted. To investigate this point a growth-chamber was set up omitting the dishes and sand but including a calibrated thermograph. A thermometer was placed at the outer surface of the cellophane.


AY
2 4 6 8 10
TUESDAY
2 4 6 8 10 NOON 2 4 6 8 10
WEDNESDAY
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THURSDAY
2 4 6 8 10 NOON 2 4 6 8 10
45
40
35
30
25
20
15
10
5
0
5
X Thermometer reading
CENTIGRADE

The chamber was placed in the greenhouse and the natural illumination was supplemented with light from a 500w. high-pressure mercury lamp.

A copy of the thermograph obtained while air was pumped through the chamber at the usual rate is shown opposite. It is seen that the higher temperatures recorded on the thermometer are 5-10° below those actually existing inside the chamber. The latter obviously acts as a trap for the heat rays and the effect is doubtless accentuated by the restricted rate of air-flow through the chamber.

Table 20. The chemical changes taking place during the ensilage of grass from the growth chamber.

Exp. No.	Material	pH	Dry matter %	per 100g. of dry matter										
				Acid (ml. $^{N}/_{1}$)			Volatile base ($ml.^{N}/_{1}$)	Nitrogen (g.)				Alcohol (g.)	Gases evolved (ml.)	
				Total	Amino	Lactic		Total	NH_2.N (% of Total)	NH_3.N (% of Total)	Increase NH_2+NH_3-N (% of Total)		CO_2	'H_2'
7	Grass	6.15	14.6	11.7	24.3	0	1.49	6.42	5.22	0.32		0.028		
	'Silage'	6.90		103.9	99.5	0	18.02		21.80	3.97	20.3	0.010	212	865
8	Grass	-	13.1		64.5	0.085	8.34	5.40	16.50	2.16		0.046	-	-
	'Silage'	6.10			95.0	0.088	43.09		24.60	11.15	17.1	0.254	77	275
9	Grass	-	13.4		-	-	-	5.62	-	-		-	-	-
	'Silage'	-			-	-	-		-	-		-	-	-
10	Grass	6.12	13.4		44.7	0.005	4.46	4.21	14.90	1.48		0.000	-	-
	'Silage	7.40			95.2	0.015	20.50		31.70	6.80	22.1	0.262	159	1080
11	Grass	6.05	20.3		16.8	0.064	1.62	4.40	5.35	0.52		0.088	-	-
	'Silage'	6.45			52.3	0.153	7.20		16.70	2.29	13.1	0.296	177	0

Table 21. Results of analyses of normal perennial rye-grass silage in tubes after incubation at 30° for 7 days.

Exp. No.	ml. N/1 acid or alkali / 100g. D.M. Total acid	Amino acid	Volatile base	pH	%N. in D.M.	Proteolysis % total N.
13	185	42	40	5.88	Young grass	-
14	167	39	20	4.60	grass just shooting	-
15	195	72	18	5.92	5.12	24.1
16	187	52	11	4.74	3.52	25.1
17	188	61	30	6.0	3.38	30.2
18	193	72	21	5.8	3.27	42.4
19	149	-	12	5.8	2.24	-
20	90	-	5.5	5.1	1.30	-
21	90	76	7.2	5.4	1.44	70.0
22	130	52	13	4.7	1.71	53.2

CHEMICAL ANALYSES OF THE GRASS FROM THE GROWTH-CHAMBER AND OF THE RESULTING SILAGE.

The chemical methods employed for the analyses were, unfortunately, not the same in all the experiments as they could only be used when they became available or after suitable modification or trial. Thus more detailed analyses could be made on the material obtained in the later work. It is necessary therefore, as well as outlining the results obtained, to indicate which methods were employed in each case.

The quantitative results are summarised in Table 20 (p.117) and qualitative information concerning the acids present in the material is given in Table 22 and Fig.20. For comparison, the results obtained by analysis of normal perennial rye-grass silage made in tubes at various times during the growing season, are shown in Table 21.

Nitrogen content. The percentage of nitrogen in the dry matter of the grass, as determined by the kjeldahl procedure (p.60), was always high, varying from 26.3% crude protein in Exp.10 to 40.1% in Exp.7. These high figures were no doubt due to the tender, leafy condition of the grass which never reached the 'shooting' stage of growth.

Total acid. This was measured in Exp.7 only, for in the later work methods were available for estimating the/

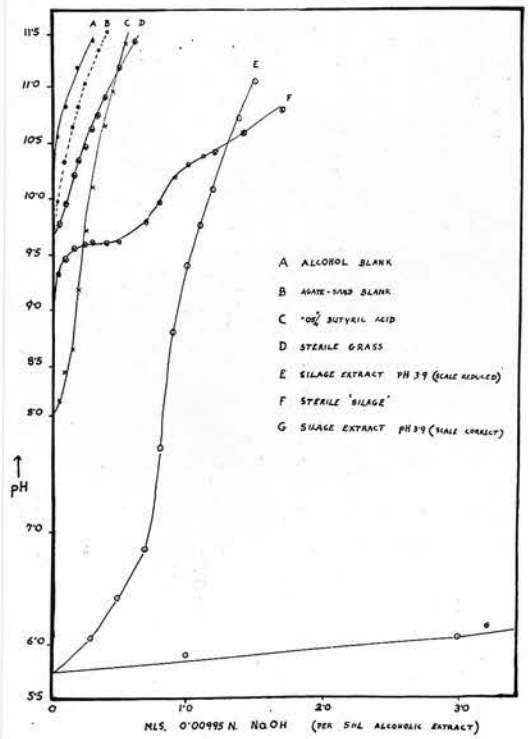


Fig. 19. Titration curves (D,F) obtained with the grass and silage extracts of Exp. 7.

the individual acids in more detail.

Alcoholic extracts, prepared from aqueous homogenates of the grass and silage were titrated to the phenolphthalein end-point (p.57). Owing to the difficulty of estimating the colour change by eye, particularly in the case of the green grass extract, the titration was carried out potentiometrically using a Cambridge pH meter and keeping the extract stirred by a stream of CO_2-free air. This allowed dilute sodium hydroxide (0.00995 N) to be used as titrant so that the titre was of reasonable magnitude considering the small amount of extract available.

The titration curves obtained are shown in Fig.19. For comparison the results of titrating a 0.05% solution of butyric acid and an extract of farm silage (pH 3.9) by this method, are also shown. The phenolphthalein end-point in these alcoholic solutions occurs at about pH 11 and the total acidity of the grass and silage extracts has been calculated from the curves on this basis.

It was important to ascertain the nature of this acidity. The position and shape of the titration curves suggested that only weak acids were involved. This was confirmed when samples of the aqueous homogenates of the grass and silage were developed/

developed on paper by the method of Hiscox & Berridge (1950); 0.2ml. of homogenate was applied as 20 x 0.01ml. spots, each of which was allowed to dry before applying the next one in the same position. No volatile acids could be detected but a partially separated spot was located, in both cases, on the starting line. Such a picture is frequently obtained when the solution under test contains amino-acids. That these were responsible in this case was shown by developing a second paper and spraying with ninhydrin when the characteristic blue colour was obtained in the same position as the partially separated spot. It was concluded that the total acidity was mostly derived from the presence of amino-acids. This conclusion was supported by the quantitative data (Table 20) for the total acidity and the 'amino-acid' acidity (p.122). The increase in total acidity which took place when the grass was ensiled was equivalent to 92.2ml. N/1 acid / 100g. dry matter compared with an increase in 'amino-acid' equivalent to 75.2ml. Also no lactic acid could be detected when 0.2ml. of the grass and silage homogenates was developed on paper by the method of Lugg & Overell (1948).

Amino-acids. The values given in Table 20 have been obtained by subtracting from the total amino-nitrogen, as determined by the method of Moore & Stein/

Stein (1948), a figure representing the amount of volatile base. They approximate therefore, to the quantities of alcohol-soluble peptides plus amino-acids. For convenience these quantities are referred to as 'amino-acids' and are expressed as percentages of the total nitrogen or as ml. $^{N}/_{1}$ acid.

The 'amino-acid' nitrogen present in the fresh grass varies from 5.2 - 16.5% of the total nitrogen; the variation presumably being due to the different periods and conditions of growth of the crops. Other workers have found that about one-sixth of the total nitrogen of plant tissue is in a soluble form and of this about 50% - or 8% of the total nitrogen - consists of amino-acids (Synge, 1951).

An outstanding feature of the increases in 'amino-acids', which were found to occur during the preparation of the 'silage', is their extreme variation. Of the factors which might be responsible for this the amount of oxygen initially present in the tube, and the moisture and nitrogen content of the grass would seem the most likely. The highest increase took place in Exp.7 where the grass had a very high nitrogen content and the greatest supply of oxygen. The variable increases which occurred in the other experiments, however, cannot be similarly explained. When the increases are expressed as a percentage of the total nitrogen their/

Table 22. The amino-acids present in alcoholic extracts of grass and 'silage' as shown by paper chromatography.

Exp. No.	7		8		10		11	
	Grass	Silage	Grass	Silage	Grass	Silage	Grass	Silage
Amino-N (g) applied	8	12	80	80	180	216	78	61
Aspartic	+	+	+	++	+	++	+	++
Glutamic	+	+	+	++	+	++	+	++
Serine	+	+	+	++	+	++	+	++
Glycine	+	+	+	++	+	++	+	++
Threonine	-	+	+	++	+	+	+	++
Alanine	-	+	+	++	+	++	+	+++
Tyrosine	-	trace	-	trace	trace	++	-	+
Histidine	-	-	-	-	-	-	-	-
Lysine	-	trace	-	+	+	+	-	+
Arginine	-	trace	-	+	+	+	-	+
* γ-Amino butyric	-	+	-	++	+	+	-	+
Proline	-	-	-	-	trace	++	-	-
Valines	-	-	-	+	+	++	-	+
Phenylalanine	-	-	-	-	-	-	-	-
Tryptophane	-	-	-	-	trace	trace	-	-
Leucines	-	+	-	-	+	++	-	+
Spot XY	+	+	+	+	+	+	+	+
Spot YX	-	-	+	trace	++	trace	+	trace
Spot Z	-	-			+	-		

R_F of unknown spots

X same as aspartic acid in butanol-acetic acid; between serine and glycine in phenol-formic acid

Y same as aspartic acid in butanol-acetic acid; between glycine and threonine in phenol-formic acid

Z same as threonine in phenol-formic acid; " " tyrosine in butanol-acetic acid

Key

\+ light spot

++ heavy spot

+++ very heavy spot

* see text

their variability is not appreciably altered. This suggests that the total nitrogen content of the grass is not an important factor in the variation.

The qualitative results obtained when alcoholic extracts of the grass and silage from the growth-chamber were developed on the chromatograms (p.85) are shown in Table 22.

Most of the individual acids could be easily identified by their relative positions and the shade of colour given by their reaction with ninhydrin. Identification was not certain, however, in the case of γ-amino butyric acid whose position on this chromatogram we have not been able to check. It seems very probable however from the 'map of spots' given by Dent (1948) and from the work of Synge (1951) that the spot has been correctly designated.

The amino-acids found in the alcoholic extracts of the fresh grass were similar to those observed in other plant juices by other workers (Steward & Thompson, 1950) and in ryegrass by Synge (1951).

As judges by the intensity of the spots, there was, in all experiments, a definite increase in the aspartic and glutamic acids, serine, glycine, threonine, tyrosine and alanine content of the silage as compared with that of the grass (Plate 14). Where the concentration of amino-acid in the initial spot was high (eg. Exp.10) lysine, arginine, γ-amino butyric/

butyric acid, valine and leucine were detected in the grass and generally increased in amount during the ensiling process. An increase in proline was also noted in one instance (Exp.10). Some unknown spots were also consistently observed; spot X was brown in colour and remained constant in intensity; spot Y, which was blue, always diminshed considerably in intensity during the incubation period (Plate 14); spot Z was only encountered in Exp.10 but may well have been found in the other experiments if the concentration of amino-nitrogen had been sufficiently high.

Volatile bases. All the estimations of volatile bases in the homogenates were done by the same method (p.85). The quantity of volatile bases in the fresh grass represented only a small fraction of the total N except for Exps. 8 & 10 where the higher values are very probably associated with the appreciable amount of dead tissue which was present in the crops.

In all experiments an increase in volatile bases took place during the ensilage process. The increases, except for that observed in Exp.11, were spectacular and comparable to those normally obtained in grass silage made in tubes (Table 21). The variability of the results is again a disconcerting feature for which no adequate explanation can be offered/

offered; the smallest increase which occurred in Exp.11 was associated with a high dry matter content of the fresh grass.

Total protein breakdown. If the summation of 'amino-acids' and volatile base is taken as a measure of protein breakdown it is seen (Table 20) that this was appreciable in all experiments during the incubation period. It ranged from 13.1% of the total nitrogen in Exp.11 to 22.1% in Exp. 10. The extent of proteolysis in normal grass silage in tubes has been found to be very variable (Table 21) and in general higher than the figures just quoted. Watson (1939, p.295) determined the decrease in copper-precipitable-nitrogen during the ensiling of young grass on a farm scale and estimated the 'true' protein breakdown at 40-45% of the crude protein; it is not possible to say what proportion of the breakdown products were soluble in alcohol. The same author, however, recorded (p.149) an increase in the soluble nitrogen of ordinary grass silage, after 8 days, amounting to about 22% of the total nitrogen; the proteolysis appeared to have reached a maximum at this time. Similar results were obtained in 1934 by Ruyter de Wildt (cited by Watson, 1939, p.301).

Lactic acid. The zero figure for Exp.7 shown in Table 20 was obtained by the chromatographic method of/

of Lugg & Overell (1950). In other experiments the colorimetric method of Barker & Summerson (1941) was employed. Although the figures for Expts. 8, 10 & 11 show an increase in lactic acid after ensiling the grass, the actual values obtained in Expts. 8 & 10 were so low as to make the estimation unreliable. No confirmation of the increase in these two cases could be obtained chromatographically by Brown's (1951) method. The increase in lactic acid in Exp.11 is considered to be reliable although again the quantity was too small to allow of visual confirmation by chromatography.

The results shown for lactic acid in Table 20 are to be compared with those obtained by Philips (1947) for barley seedlings which had respired anaerobically for 6 hr. (p. 33). The smallest increase she recorded is equivalent to about 2.3 ml. N/1 acid / 100 g. dry matter assuming that the dry matter of her samples was about 20%. The lactic acid content of ordinary grass silage in tubes after 7 days at 30° varies according to the rate at which acidification is taking place. Thus values of 10 ml. N/1 (pH 5.76) and 108 ml. N/1 (pH 3.8) have been recorded.

Other non-volatile acids. The qualitative results obtained by developing ether extracts of the grass and silage homogenates (p. 75) by the method of Brown (1951) are shown diagrammatically in Fig. 20; the/

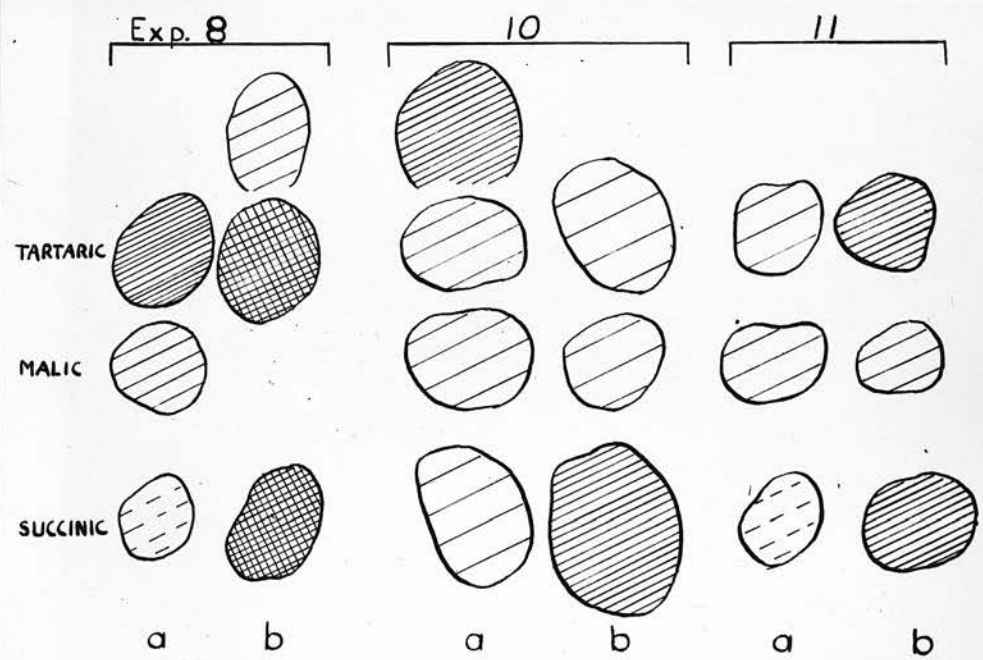


Fig. 20. Diagramatic representation of chromatograms of non-volatile acids present in grass(a) and silage(b) of exps. 8, 10, 11. The intensity of the spots is represented by the density of the shading.

the size and shape of the separated spots were traced from the actual chromatograms. In all cases the positions of the spots were checked against solutions of known acids.

One consistent feature is the quite definite increase in succinic acid which takes place during the incubation period. In Exp.8 it was interesting to note that the disappearance of malic acid on incubation coincided with the appearance of an unidentified acid above tartaric. The unknown acid was again observed in Exp.9, this time in the fresh grass. In Expts. 8 and 11 the silage gave much more intense spots of tartaric acid than did the grass.

In over twenty examinations of normal silage, made of perennial rye-grass in tubes, an increase in succinic acid has always been noted. It has been demonstrated after incubation for one day at 30°. Plate 15 shows the heavy spot of succinic acid obtained from normal tube silage of perennial rye-grass after incubation for ten days. A further illustration of this increase is to be found on Plate 9. ~~(p. —)~~.

Volatile acids. The chromatographic method of Hiscox & Berridge (1950) was used throughout (p. 66). In none of the experiments could any volatile acids be demonstrated in the grass or silage homogenates by applying them directly to the paper strip. To improve/

improve the sensitivity of the test, therefore, an aqueous solution of the organic acids was obtained by ether-extraction and distillation (p.75) thereby increasing the acid concentration about twenty times; 1ml. of the aqueous solution then represented approximately 0.4g. dry matter of the grass or silage. This concentrated solution was then developed on the chromatogram. In all experiments no fatty acids higher than acetic acid could be detected. An increase in acetic acid was however, noted in the silage of Expts. 8, 10, 11 (Table 23).

Table 23. The acetic acid present in extracts of the grass and 'silage' from the growth chamber as determined qualitatively on the paper chromatogram of Hiscox & Berridge (1950)

	Volume of extract applied to paper (ml.)					
Exp. No.	7		8		10	11
	0.01	0.01	0.02	0.03	0.01	0.01
Grass	0	0	0	slight	slight	0
Silage	0	0	0	distinct	distinct	very distinct

In Exp.11 in which the greatest increase was recorded the intensity of the chromatogram spot was compared with that of spots containing known amounts of acid; it was estimated in this way that the acetic acid content of the silage was about 0.03% of the fresh material. The quantity of acetic acid present/

present in the silage of Expts.8 and 10 was considerably smaller than this. As can be seen from the Table 23 an increase was only just detectable in Exp.8. The negative result obtained in Exp.7 may have been due to the use of too small a quantity of extract.

Hydrogen ion concentration. The pH of the homogenates of the grass was about 6.1 units, a value which has been frequently recorded for normal grass crops. On incubation an appreciable increase in pH took place in Expts. 7, 10 and 11 and it appears to be related to the quantity of volatile base present, particularly if this is expressed as a percentage of the total N. Such a relationship, however, appears to be doubtful in the case of Exp.8 in which the largest amount of volatile bases was found but, unfortunately, in this case the pH of the grass was not obtained.

Alcohol. In Expts. 7-10 the alcohol content was determined on 1ml. of homogenate. The more sensitive method using 0.1ml. was used in Exp.11.

The significance to be attached to the result obtained in Exp.7 is doubtful for the homogenate used was very dilute owing to the small yield of crop. For the remaining experiments, however, the increase in alcohol content to a value of 0.25-0.30% of the dry matter is convincing.

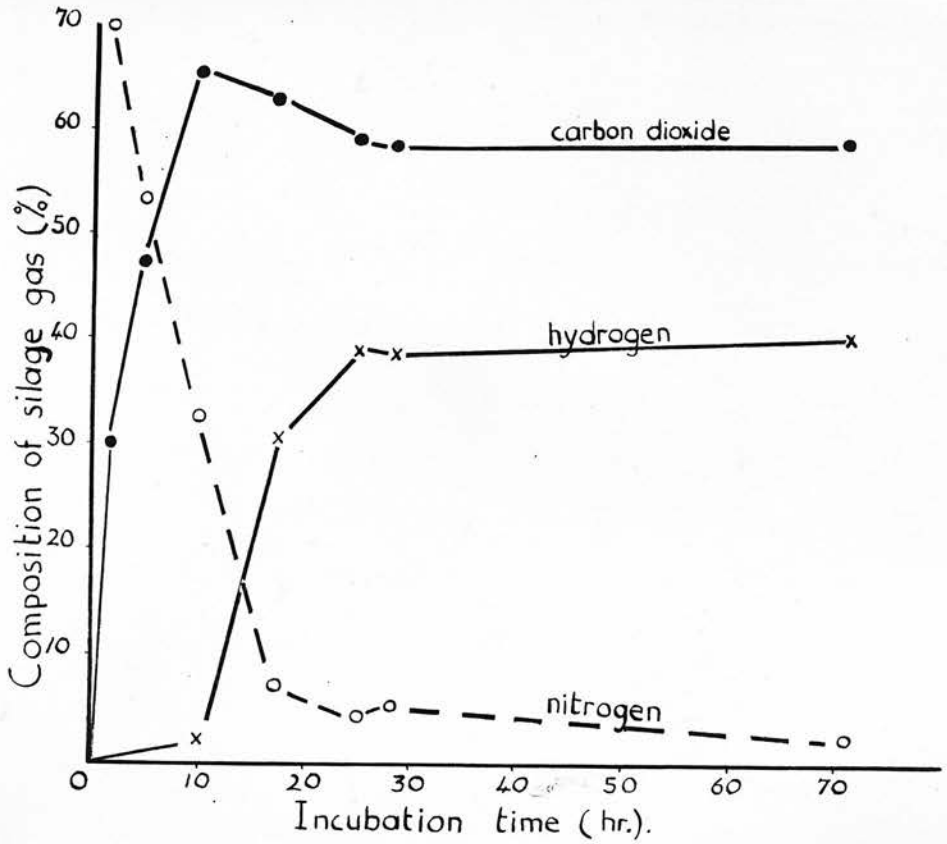


Fig. 21. Typical results obtained by analysis of gases from normal grass silage in tubes.

Alcohol in normal grass silage in tubes has been measured in only a few cases after seven days incubation at 30° when the quantity found was just over 0.3%. Watson (1939, p.143) quotes an average alcohol content of fresh grass silage as about 0.3%. Peterson et al. (1925, p.16) found 0.525% alcohol in maize silage after seven days.

Gases evolved. In Expts. 7, 8, 10 the gases evolved were estimated by the approximate method which suffers from the disadvantages mentioned on p.59 It seems fairly certain, however, from accurate analyses which have been carried out on numerous samples of gases from ordinary grass silage in tubes, that only carbon dioxide and hydrogen are normally present. Fig.21 shows a typical result; the gases liberated from one silo were collected in seven samples over a period of 4 days at the end of which gas evolution had practically ceased. It is noteworthy that carbon dioxide is liberated from almost the beginning of the incubation. Hydrogen on the other hand is present in appreciable quantity only after 10-20hr. incubation suggesting that it arises as a result of bacterial activity.

The rates at which 'hydrogen' was liberated in Expts. 7, 8, 10 are shown in Fig.22. The total volume of the silo tubes used was 30ml. and it is possible to calculate the approximate amount of nitrogen/

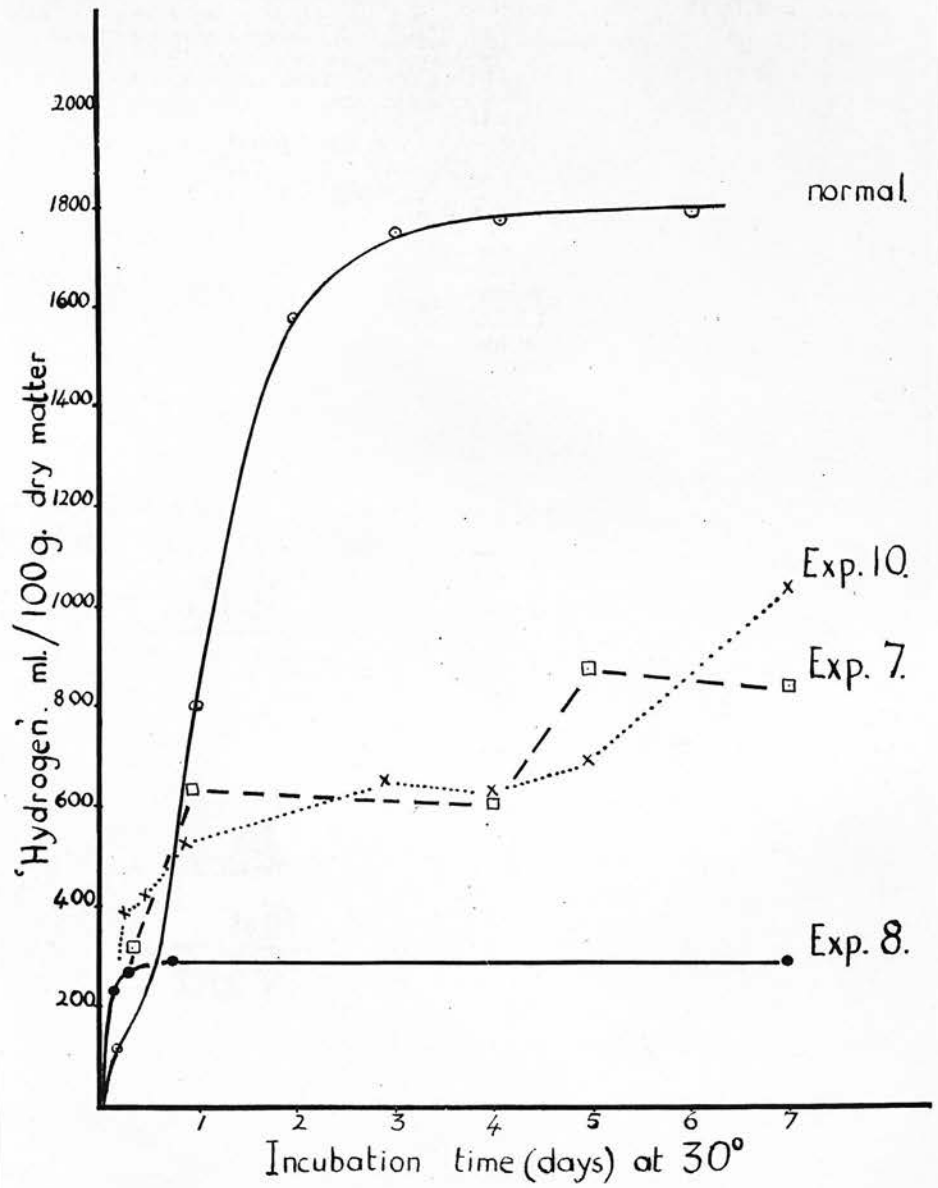


Fig.22. Rate of evolution of 'hydrogen' from sterile and normal grass silage.

nitrogen present in the tube at the start of the incubation period assuming the specific gravity of the grass to be about unity. The results calculated to 100g. of dry matter are: Exp.7, 1970ml.; Exp.8, 765ml.; Exp.10, 840ml. Thus, taking into account the limitations of the method, all the 'hydrogen' which was recorded, could have been nitrogen displaced from the silo tube as a result of plant respiration. The steep rise in the curve for Exp.10 between the fifth and seventh day is, however, disconcerting and difficult to explain. In nine experiments with normal grass silage the mean evolution of 'hydrogen' measured in the same way was 1830ml. (minimum 1500ml.) and in all cases the shape of the curve was similar. The results obtained in one experiment are included for comparison in Fig.22.

It was apparent that while the results from Exp.7, 8, 10 were suggestive they were inconclusive, and in the final experiment (Exp.11) the gases evolved were collected over mercury using the apparatus illustrated in Fig.12 (p.59). The first bubble of gas came over after 3 hr. incubation at 30° and after 7 hr. incubation the total volume was only 2ml. No further gas evolution took place. On analysis only carbon dioxide and nitrogen were found, the former representing 20.2% of the total. A further gas sample was obtained from the silage tube by placing it under reduced pressure; again only carbon dioxide (20.0%) and nitrogen could be detected.

DISCUSSION OF RESULTS

The growth-chamber

In its present form the chamber is not entirely satisfactory. The main difficulty is to make a good joint with cotton-wool between the upper framework and the base part; this is not an easy operation because of the unevenness of the metal strip used to make the framework and also because, as at present constructed, the framework does not make a perfect fit with the base-part. Also the cellophane used for covering the framework is in many ways unsuitable; it contracts on sterilizing, is easily punctured and does not permit a perfect seal to be made at the end-folds.

These disadvantages would be overcome if the upper part was a glass tank. It would then be possible to make an excellent joint by standing the inverted tank on cotton wool in a narrow metal trough permanently attached to the base-part (Fig. 23).

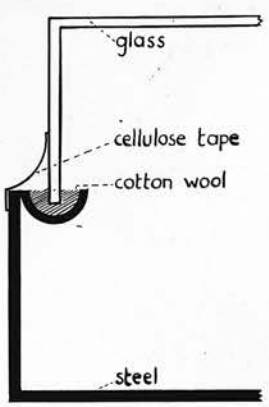


Fig. 23. Section of growth-chamber showing a suggested modification in construction and assembly.

As previously mentioned (p.45) it has not been possible to obtain a suitable tank in heat-resistant glass/

glass. A tank of soda-glass measuring 18 x 12 x 12 in., which was available commercially, was cut down to the required depth but would not withstand autoclaving. If economic considerations preclude the possibility of obtaining a heat-resistant glass top an alternative procedure would be to sterilise the base-part and its contents as before, fit on a chemically cleaned soda-glass top and sterilise the complete assembly by injecting liquid ethylene oxide. Preliminary experiments have shown that a short contact with the vapour of this liquid is lethal to timothy seed. Consequently introduction of the seed would need to follow later.

It is considered that no risk of contamination is involved when the air-filter and water-reservoir are connected to the base-part if the connections are carefully made. The efficiency of the filter under the conditions used cannot be questioned on the basis of the results of the sterility tests which were applied. Nevertheless, the packing of cotton-wool into a filter, in such a way as to avoid channeling, is not easy (Kluyver & Visser, 1950) and an alternative packing such as carbon powder might be considered. A chance of contamination is introduced by the technique employed in stopping the water siphon; when the reservoir is lowered below the level of the water-inlet/

inlet, air is sucked into the chamber through the cotton-wool joint between the base-part and the upper framework, and micro-organisms might penetrate the joint at any weak spot.

After the complete assembly has received its final sterilization the interior of the chamber is wet with condensate. The moisture, however, quickly disappears when the air-flow is started. The cotton-wool used for the joints has never been observed in a wet condition and mould growth has never been noted on its surface.

Preparation of microbe-free seed and its introduction into the chamber

The procedure described (p. 37) for disinfecting timothy seed, when checked by the more sensitive test for sterility (p.109), appears to be entirely satisfactory. There is little risk of contamination occurring while the seed is being introduced into the chamber.

Growth conditions inside the chamber

It is recognised that much work requires to be done to ascertain the best conditions for the growth of timothy inside the sterile chamber.

The nutrient solution used, while satisfactory for plant growth outside of the chamber, may need modification to suit the abnormal conditions inside.

The rate of air-flow was selected quite arbitrarily and the carbon dioxide available may be a limiting factor for growth. Probably of greater importance is the relationship between flow-rate and temperature. It seems certain (p.115) that the temperature inside the chamber has been allowed to rise too high during the growth period and it is considered most important that future work should aim at controlling this factor. Increased rates of air-flow should help to keep the temperature down but would increase the strain on the filter. The most satisfactory arrangement would seem to be the use of artificial illumination only; high pressure mercury vapour lamps with some type of heat filter, or preferably fluorescent lamps, would provide adequate means of temperature control.

If the modifications are made to the growth-chamber and to the environment inside the chamber it seems likely that completely microbe-free crops could be obtained. The apparatus would then prove of great use, not only in studies similar to those described in this thesis, but also for investigations in plant physiology; it would be useful, for instance, in researches into plant respiration (p.17, 25).

Bacteriological aspects

In none of the experiments was it possible to grow/

grow a crop of grass free from micro-organisms. The nearest approach to this condition was achieved in Exp. 7 where only mould could be obtained by enrichment from the grass but could not be found by direct plating of the 'silage homogenate'. In this experiment, therefore, it is reasonably certain that no products of microbial origin were present in the 'silage'.

The contaminants found in Exp.8 & 10 were also strictly aerobic. Since anaerobic conditions are rapidly established in the silo tubes extensive microbiological changes seem to be precluded. In Exp. 8 the fungal count suggests that little if any mould growth occurred during the ensilage period. No certain statement can, however, be made with regard to Exp. 10 for here the numbers of contaminants were high and no accurate estimate is available of the numbers initially present on the fresh grass.

The facultative nature of one of the contaminants present in Exp. 11 throws doubt on any interpretation of the results of this experiment.

It is unfortunate that the origin of the contamination is not known for certain. All the evidence available suggests that the joint between the base-part and the upper framework is probably at fault. The method of overcoming this defect has already been discussed.

Chemical changes during the ensilage process

When interpreting the data obtained from analysis of the tube 'silage' two facts have to be borne in mind; firstly, the bacteriological aspects which have justbeen discussed and secondly, that since no observations have been made on normal silage made from timothy grown under conditions similar to those existing in the chamber, it has only been possible to make comparisons with normal perennial rye-grass silage made in tubes.

It is quite clear from the results that considerable protein breakdown can take place as a result of plant cell metabolism. The extent of proteolysis approaches that which has been found to occur in normal grass silage. This finding is in agreement with that of the majority of those workers who have ensiled plants in the presence of anti-septics (p. 3). As arresult of the proteolysis an increase in the concentration of most of the common amino-acids takes place. If the growth of any species of micro-organism in normal timothy silage is restricted by the short supply of any amino-acids it seems, from the qualitative evidence, that only one or two (e.g. tryptophane) are likely to be involved.

An increase in volatile base to a level similar

to/

to that reached in normal silage was a constant feature in all experiments. That the plant cells of detached leaves possess the enzymes necessary for the breakdown of protein to amide and ammonia has been shown by Yemm (1949-50) for barley and by Vickery (1938) for tobacco. Again Virtanen (p. 7) considered amide to be the source of ammonia in A.I.V. silage.

It is generally assumed that the formation of volatile bases in silage is a result of bacterial activity. The data obtained from the tube 'silage', however, suggests that the activity of the plant cell is responsible for much of the volatile base formed. If this is so it will be of importance to ascertain the factors governing the extent of this process which, in the early period of silage making, may be responsible for a considerable buffering effect. In practice, however, it is to be expected that the contribution made by plant enzyme activity to the overall protein breakdown will be less than is indicated by the results from tube 'silage', for the acidification which takes place in normal silage should, if sufficiently rapid, reduce the activity of these agents (p. 8).

There have been conflicting reports from previous workers (p.31) as to whether lactic acid is/

is a product of the anaerobic metabolism of leaf tissue. My results appear to indicate that very small amounts of lactic acid may be formed by the metabolism of plant cells during the ensilage process but the evidence is inconclusive and, in any case, the quantity of acid involved is negligible. The qualitative evidence that cells of grass tissue can produce succinic acid is, however, impressive but, in the absence of quantitative information, its possible significance is not known. The presence of this acid was noted in maize silage by Annett & Russell (1908) but it has received little attention since; Common (1941) found that its presence in silage could be detected from the shape of a buffer index curve of a grass silage extract.

No generalisation can be made about the changes which took place in the amounts of other non-volatile carboxylic acids. The disappearance of malic acid during the incubation period is in agreement with the observation of Pepkowitz et al. (1944) but is at variance with the results of Wood et al. (1943) (p. 28).

It is impossible to assess the significance of the increases in acetic acid which occurred in the tube 'silage'. In Exp. 11 where the greatest increase was recorded the metabolism of the corynebacterium present (p.111) is probably a sufficient/

sufficient explanation. Nevertheless it is true to say that in no case was the quantity of acetic acid significant from the viewpoint of silage making.

The alcohol production during the incubation period in Exp. 8, 10, 11 were very similar and approached the average value of about 0.3% which is found in normal silage. The microflora present in Exp. 8,&10 was strictly aerobic and it seems reasonable to infer that in these instances, at any rate, the alcohol was a product of plant cell metabolism. In Exp. 7 a convincing increase in alcohol content was not recorded; the small amount of material available for testing and the comparatively low sensitivity of the method of estimation in this case, may be a sufficient explanation.

Little interest appears to have been taken in the composition of silage gases. Peterson et al. (1925) showed that the gases from maize silage consisted solely of carbon dioxide and nitrogen, the latter being derived from the air which was initially present in the silo. It has been generally assumed since that time that the gases from grass silage have a similar composition. Thus Watson (1939, p.131) collected the gases from grass silage over water containing a layer of oil on/

on its surface and constructed graphs showing the volume of carbon dioxide evolved during the ensilage process. It is quite certain, however, that large amounts of hydrogen are evolved from normal perennial rye-grass silage (Fig.21); sufficient hydrogen can be collected in a tube to allow an appreciable explosion to take place on ignition. There is no suggestion that this hydrogen arises as a result of plant cell activity; in support of this view the data of Exp. 11 are regarded as conclusive as it is unlikely that the contaminants present could have seriously influenced the negative result obtained.

Anaerobic conditions are established very quickly in the tubes of 'silage'. Thus in the analysis of gases from perennial rye-grass silage no oxygen could be detected in a sample collected in the first 2½ hr. It seems reasonable to take the first bubble of gas evolved as an indication of the end of the aerobic phase for during this period the respiratory coefficient is below unity. (Peterson et al. 1925). On this basis, and also as a result of gas analysis, no oxygen was present in the tube of Exp. 11 after 3 hr. This result is in general agreement with previous work (p. 24).

The extent to which the chemical changes in microbe-free 'silage' occur in the aerobic or in the/

the anaerobic phase is not known; if it could be determined the result might be of practical value. Previous workers (p. 21) have suggested that the formation of amino-acids, amides and ammonia by plant cells are aerobic processes, unless the tissues are extensively damaged when appreciable proteolysis may take place under anaerobic conditions. The rapid establishment of anaerobic conditions in the tube silos suggests, therefore, that tissue damage is, in this case, the main factor. In practice, however, the time elapsing from cutting the fodder to the establishment of anaerobiosis may be also of considerable importance.

It is realised that the conclusions drawn from the results presented in this thesis can only be tentative since the grass used for ensiling was not completely microbe-free; indeed, had the tests for sterility been negative the possibility exists that some micro-organisms were present but could not be cultivated. Nevertheless the approach which has been used appears worthy of repetition and development. For instance, it should make possible the study of the changes in silage brought about by pure cultures of micro-organisms. A disadvantage of the technique described is that it does not appear possible to grow timothy to maturity since the height of the growth-chamber is limited by the size/

size of the autoclave available. Consequently investigations are restricted to young grass. This difficulty should not be insuperable.

SUMMARY

A growth-chamber is described in which attempts have been made to grow microbe-free timothy grass from which silage can be made without chance of contamination. It was expected that useful information about the chemical changes due to plant cells in the ensilage process would result from analyses of the products.

The attempts were only partially successful; sterile silage was produced in one case only but some tentative conclusions could be drawn from the results of other experiments in which only a very restricted microflora was present.

As a result of plant cell metabolism the protein breakdown, production of volatile base and possibly of alcohol occurred to an extent comparable to that observed in normal silage. No significant increase in lactic or volatile acids took place but a definite increase in succinic acid was noted although no quantitative data were obtained. Hydrogen, which is normally evolved by grass silage in tubes, does not arise as a result of plant cell activity.

New methods are presented for the quantitative estimation of volatile base and alcohol in silage and qualitative methods of analysis are described for amino-acids, volatile acids and other carboxylic acids.

It is considered that the growth-chamber can be further improved in design and with the technique described should prove of use in other similar studies to those reported and also in other plant physiological investigations.

REFERENCES

Allen, L.A., Harrison, J., Watson, S.J. & Fergusson, W.S. (1937). A study of the chemical and bacteriological changes in grass silage. J. agric. Sci. 27, 271.

Annett, H.C. & Russell, E.J. (1908). The composition of green maize and of the silage produced therefrom. J. agric. Sci. 2, 382.

Ash, C.G. & Allen, O.N. (1948). A comparison of methods recommended for the surface sterilisation of leguminous seeds. Soil Sci. Soc. Amer. Proc. 13, 279.

Ashton, F.L. (1936). Selenium as a catalyst in the Kjeldahl method as applied to soil and grass analysis. J. agric. Sci. 26, 239.

Aubert, M.E. (1892). Recherches sur la respiration et l'assimilation des plantes grasses. Rev. gen. Bot. 4, 321.

Audus, L.J. (1935). Mechanical stimulation and respiration in the Cherry-Laurel. New. Phytol. 34, 386.

Babcock, S.M. & Russel, H.L. (1902). Die bei der Herstellung von Gärfutter (Silage) wirkenden Ursachen. Zbl. Bakt. (2 Abt.) 9, 81.

Barker, S.B. & Summerson, W.H. (1941). The colorimetric determination of lactic acid in biological material. J. biol. Chem. 138, 535.

Barnett, A.J.G. (1951). The colorimetric determination of lactic acid in silage. Biochem. J. 49, 527.

Bartholomew, W.V. & Broadbent, F.E. (1949). Apparatus for control of moisture, temperature and air composition in microbiological respiration experiments. Soil Sci. Soc. Amer. Proc. 14, 156.

Bennet-Clark, T.A. (1937). Organic acids of plants. Ann. Rev. Biochem. 6, 579.

Blackman, F.F. & Parija, P. (1928). The respiration of a population of senescent ripening apples. Proc. Roy. Soc. B 103, 412.

Blackman, G.E. & Templeman, G. (1940). The relation of light intensity and nitrogen supply to the protein metabolism of the leaves of grasses. Ann. Bot. 4, 533.

Brown, F. (1951). Application of paper chromatography to the separation of non-volatile carboxylic acids. Nature, Lond. 167, 441.

Chibnall, A.C. (1939). Protein Metabolism in the Plant. London : Oxford University Press.

Clagett, C.O., Tolbert, H.E. & Burris, R.H. (1949). Oxidation of α-hydroxy acids by enzymes from plants. J. biol. Chem. 178, 977.

Common, R.H. (1941). The buffer curves of silage extract. Analyst 66, 407.

Conway, E.J. (1947). Microdiffusion Analysis and Volumetric Error. 2nd. ed. London : Crosby Lockwood & Son, Ltd.

Conway, E.J. (1950). Microdiffusion Analysis and Volumetric Error. 3rd. ed. London : Crosby Lockwood & Son, Ltd.

Cruickshank, D.H. & Wood, J.G. (1944). The metabolism of starving leaves. 5. Changes in amount of some amino-acids during starvation of grass leaves. Aust. J. exp. Biol. med. Sci. 22, 111.

Cruickshank, D.H. & Wood, J.G. (1945). The metabolism of starving leaves. 6. Nitrogen balance sheet and changes in organic acid content during starvation of oat leaves. Aust. J. exp. Biol. med. Sci. 23, 243.

Cunningham, A. & Smith, A.M. (1939). The microbiology of silage made by the addition of mineral acids to crops rich in protein. Zbl. Bakt. (2 Abt.) 100, 394.

Davidson, J. (1949). The colorimetric determination of lactic acid in milk and milk products. J. Dairy Res. 16, 209.

Dent, C.E. (1948). A study of the behaviour of some sixty amino-acids and other ninhydrin-reacting substances on phenol-'collidine' filter paper chromatograms. Biochem. J. 43, 169.

Duff, G.H. & Forward, Dorothy F. (1949). The respiration of the seedling wheat leaf in starvation and ontogeny. Canad. J. Res. C 27, 125.

Dyer, D.C. (1917). A new method of steam distillation for the determination of the volatile fatty acids including a series of qualitative colorimetric reactions for their identification. J. biol. Chem. 28, 445.

Elsden, S.R. (1946). The application of the silica gel partition chromatogram to the estimation of volatile fatty acids. Biochem. J. 40, 252.

Esten, W.T. & Mason, C.J. (1912). Silage fermentation. Storrs (Conn.) agric. Exp. Sta. Bull. No. 70.

Flood, A.E., Hirst, E.L. & Jones, J.K.N. (1948). Quantitative analysis of mixtures of sugars by the method of partition chromatography. J. chem. Soc. p.1679.

Foreman, F.W. (1920). Rapid volumetric methods for the estimation of amino-acids, organic acids and organic bases. Biochem. J. 14, 451.

Foreman, F.W. (1928). Further developments of rapid volumetric methods for the determination of amino-acids, organic acids and bases. Biochem. J. 22, 208.

Forward, Dorothy F. (1952). The respiration of barley seedlings in relation to oxygen supply. New Phytol. 50, 297.

Franzen, H. & Stern, E. (1921). Über das Vorkommen von Äthylidenmilchsäure in den Blattern der Himbeere (Rubus Ideaeus). Hoppe-Seyl. Z. 115, 270.

Fred, E.B., Baldwin, I.L. & McCoy, E. (1932). Root Nodule Bacteria and Leguminous Plants, p.266-7. Madison.

Friedemann, T.E. (1938). The identification and quantitative determination of volatile alcohols and acids. J. biol. Chem. 123, 161.

Fry, G. (1885). Sweet Silage. London : Agricultural Press Co.

Fürth, O. (1925). Abderhaldens Handbuch der biologischen Arbeitsmethoden. (1. Abt.) T. 6, S. 758.

Gerlach, Günther & Seidel (1927). Einsäuerungsversuche mit grünen Lupinen und grüner Serradella. Zbl. Bakt. (2. Abt.) 69, 243 & 247.

James W. O. & Bunting (1941). New Phytologist 40, 268.

James, Heard & James. (1944). New Phytologist 43. 62.

Goddard, D.R. & Meuse, B.J.D. (1950). The respiration of higher plants. Ann. Rev. Plant Physiol. 1, 207.

Greenberg, D.M. & Winnick, T. (1945). Enzymes thhat hydrolyse the carbon-nitrogen bond: proteinases, peptides and amidases. Ann. Rev. Biochem. 14, 31.

Harding, V.J. & Maclean, R.M. (1916). The ninhydrin reaction with amines and amides. J. biol. Chem. 25, 337.

Hiscox, E.R. & Berridge, N.J. (1950). Use of paper partition chromatography in the identification of the volatile fatty acids. Nature, Lond. 166, 522.

Holden, M.&Tracey, M.V. (1948). The effect of fertilizers on the level of nitrogen, phosphorous, protease and pectase in healthy tobacco plants. Biochem. J. 43, 147.

Hunter, C.A. (1921). Bacteriological and chemical studies of different kinds of silage. J. agric. Res. 21, 767.

Hunter, O.W. (1917). Micro-organisms and heat production in silage fermentation. J. agric. Res. 10, 75.

Hunter, O.W. (1918). Bacteriological studies on alfalfa silage. J. agric. Res. 15, 571.

Hunter, O.W. & Bushnell, L.D. (1916). The importance of Bacterium bulgaricus group in ensilage. Science N.S. 43, 318.

James, A.T. & Martin, A.J.P. (1952). Gas-liquid partition chromatography: the separation and micro-estimation of volatile fatty acids. Biochem. J. 50, 679.

James, W.O. (1946). The respiration of plants. Ann. Rev. Biochem. 15, 417.

James, W.O. & Cragg, J.M. (1943). The ascorbic acid system as an agent in barley respiration. New Phytol. 42, 28.

Keddie, R.M. (1952). The enumeration of Lactobacilli on grass and in silage. Proc. Soc. app. Bact. (in press).

Kidd, F., West, C. & Briggs, C.E. (1921). A quantitative analysis of the growth of Helianthus annuus. Part 1. The respiration of the plant and its parts throughout the life cycle. Proc. Roy. Soc. B 92, 368.

Kluyver, A.J. & Visser, J. (1950). Some observations on air filtration. Ant. v. Leeuwenhoek, 16, 311.

Lamb, A.R. (1917). The relative influence of micro-organisms and plant enzymes on the formation of corn silage. J. agric. Res. 8, 361.

Leach, W. (1936). The relation between the respiration in air and in nitrogen of certain seeds during germination. Proc. Roy. Soc. B 119, 507.

Lechartier, G. & Bellamy, F. (1869). De la fermentation des fruits. C. R. Acad. Sci., Paris 69, 466.

Lighting Service Bureau, (1950). Electric Lamps. London : Spottiswoode, Ballantyne & Co. Ltd.

Ludwig, C.A., Allison, F.E., Hoover, S.R. & Minor, F.F.W. (1940). The production and utilisation of alcohol by plant tissue. Science 91, 171.

Lugg, J.W. & Overell, B.T. (1948). One and two-dimensional partition chromatographic separation of organic acids an an inert paper sheet. Aust. J. Sci. Res. A 1, 98.

Marsh, P.B. & Goddard, D.R. (1939). Respiration and fermentation in the carrot (Daucus carota). Amer. J. Bot. 26, 724 & 767.

Miller, E.C. (1931). Plant Physiology, p.735. New York & London : McGraw Hill Book Co. Inc.

Miller, E.C. (1938). Plant Physiology, p. 939. New York & London : McGraw Hill Book Co. Inc.

Moore, S. & Stein, W.H. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. J. biol. Chem. 176, 367.

Pasteur, L. (1872). Faits nouveaux pour servir a la connaissance de la theorie des fermentations. C. R. Acad. Sci., Paris 75, 784.

Peterson, W.H. & Fred, E.B. (1920). The role of pentose-fermenting bacteria in the production of corn silage. J. biol. Chem. 41, 181.

Peterson, W.H., Hastings, E.G. & Fred, E.B. (1925). The principle changes which take place in the making of silage. Agric. Exp. Sta. Wis. Res. Bull. No. 61.

Pucher, G.W. & Vickery, H.B. (1934). Determination of the acids in plant tissue. Industr. Engng. Chem. Anal. 6, 190.

Pucher, G.W. & Vickery, H.B. (1941). Determination of succinic acid in plant tissue. Industr. Engng. Chem. Anal. 13, 412.

Pepkowitz, L.P., Gilbert, S.G. & Shive, S.W. (1944). The importance of oxygen in the nutrient substrate of plants. Soil Sci. 58, 295.

Philips, Jean W. (1947). Studies on fermentation in rice and barley. Amer. J. Bot. 34, 62.

Philips, P.R. & Kaye, S. (1949). The sterilizing action of gaseous ethylene oxide. Amer. J. Hyg. 50, 270 - 306.

Pucher, G.W. & Vickery, H.B. (1941). Succinic acid as a metabolite in plant tissue. Plant Physiol. 16, 771.

Rahn, O. (1910). The usefulness of curves in the interpretation of microbial and biochemical processes. Mich. Agric. Exp. Sta. Tech. Bull. No. 5.

Reid, R.L. & Lederer, M. (1951). Separation and estimation of C_2-C_7 fatty acids by paper partition chromatography. Biochem. J. 50, 60.

Reynier, J.A. (1943). Micrurgical and Germ-Free Methods. Illinois : C.C. Thomas.

Richter, D. (1937). Adrenaline and amine oxidase. Biochem. J. 31, 2022.

Roberts, D.W.A. (1951). The effects of starvation and mechanical stimulus on the respiratory metabolism of the first leaf of the wheat plant. Canad. J. Bot. 29, 383.

Rosenberger, R.F. (1952). The development of methods for the study of obligate anaerobes in silage. Proc. Soc. app. Bact. (in press).

Russell, E.J. (1908). The chemical changes taking place during the ensilage of maize. J. agric. Sci. 2, 392.

Russell, J.A. (1944). Interference with the colorimetric determination of lactic acid (Barker-Summerson method) by nitrate and nitrite ions. J. biol. Chem. 156, 463.

Ryan, M.T., Nolan, J. & Conway, E.J. (1948). Micro-diffusion methods. Improvement in the determination of carbon monoxide and alcohol. Biochem. J. 42, lxiv.

Saifi, A.F. & Barker, J. (1939). The respiratory metabolism of potatoes under anaerobic conditions. Report of the Food Investigation Board, D.S.I.R., p. 84.

Schneider, A. (1941). Über das Auftreten der Milchsäure in höheren Pflanzen insbesondere während der Keimung. Planta 32, 234.

Smith, A.M. (1938). The determination of organic acids in silage extracts and bacterial cultures. Analyst 63, 777.

Smith, A.M. & Agiza, A.H. (1951). The determination of amino-acids colorimetrically by the ninhydrin reaction. Analyst 76, 623.

Steward, F.C. & Thompson, J.F. (1950). The nitrogenous constituents of plants. Ann. Rev. Plant Physiol. 1, 233.

Stich (1891). Die Athmung der Pflanzen bei verminderter Sauerstoffspannung und bei Verletzungen. Flora 74, 1.

Stiles, W. (1946). Respiration II. Bot. Rev. 12, 165.

Stiles, W. & Leach, W. (1932). Respiration in Plants. London : Methuen & Co. Ltd.

Sumner, J.B. & Somers, G.F. (1947). Chemistry and Methods of Enzymes. 2nd. ed. New York : Academic Press Inc.

Synge, R.L.M. (1951). Non-protein nitrogenous constituents of rye-grass. Biochem. J. 49, 642.

Thimann, K.V. & Bonner, W.D. Jr. (1950). Organic acid metabolism. Ann. Rev. Plant Physiol. 1, 75.

Thomas, M. (1947). Plant Physiology. 3rd. ed. London : Churchill Ltd.

Thomas, M. & Fidler, J.C. (1933). Studies in zymasis. VI. Zymasis by apples in relation to oxygen concentration. Biochem. J. 27, 1629.

Thomas, M. & Fidler, J.C. (1941). Studies in zymasis. VIII. The discovery and investigation of aerobic HCN zymasis in apples treated with hydrogen cyanide. New Phytol. 40, 217.

Tiemann & Rehm (1931). Die Futterkonservierung im Silo mit luftdichtem Deckelverschluss. Landw. Jb. 74, 47-63.

Tracey, M.V. (1948). Leaf proteases of tobacco and other plants. Biochem. J. 42, 282.

Trautwein, K. (1928). Zur Biologie der Grunfütterkonservierung. Zbl. Bakt. (2. Abt.) 74, 1.

Turner, J.S. (1938). The respiratory metabolism of carrot tissue. Materials and Method. New Phytol. 37, 232.

Vickery, H.B. & Pucher, G.W. (1940). Organic acids of plants. Ann. Rev. Biochem. 9, 529.

Vickery, H.B. (1938). The metabolism of protein in green leaves. Cold Spring Harbor Symposia on Quantitative Biology, 6, 67.

Virtanen, A.I. (1933). The A.I.V. method for the preservation of fresh fodder. Acta chem. fenn. A 6, 1.

Virtanen, A.I. (1936). The A.I.V. process in ththeory and practice. Monthly Bulletin of Agricultural Science and Practice, No. 10, Rome, International Institute of Agriculture.

Virtanen, A.I. (1937). The microbiology of ensilage production. Zbl. Bakt. (2. Abt.) 95, 472.

Watson, S.J. (1939). The Science and Practice of Conservation : Grass and Forage Crops. Vol.1. London : "The Fertilizer and Feeding Stuffs Journal", 16, Mark Lane, E.C.3.

Watson, S.J. & Fergusson, W.S. (1937). The chemical composition of grass silage. J. agric. Sci. 27, 1.

Widmark, E.M.P. (1922). Eine Mikromethode zur Bestimmung von Äthylalkohol im Blut. Biochem. Z. 131, 473.

Wiegner, G. (1926). Anleitung zum quantitativen agriculturchemischen Praktikum. Berlin : Gebruder Borntraeger.

Winnick, T. (1942). Determination of ethyl alcohol by micro-diffusion. Industr. Engng. Chem. Anal. 14, 523.

Woodman, H.E. (1925). Critical examination of the methods employed in silage analysis. J. agric. Sci. 54, 343.

Wollny (1897). Die Zersetzung der organischen Stoffe. cited by Russell, E.J. (1908).

Wood, J.G., Cruickshank, D.H. & Kuchel, R.H. (1943). The metabolism of starving leaves. Aust. J. exp. Biol. med. Sci. 21, 37.

Woodman, H.E. & Amos, A. (1926). The losses in the tower silo. J. agric. Sci. 16, 539.

Yemm, E.W. (1935). The respiration of barley plants. II. Carbohydrate concentration and carbon dioxide production in starving leaves. Proc. Roy. Soc. B 117, 504.

Yemm, E.W. (1937). The respiration of barley plants. III. Protein catabolism in starving leaves. Proc. Roy. Soc. B 123, 243.

Yemm, E.W. (1949-50). The respiration of barley plants. IV. Protein catabolism and the formation of amides in starving leaves. Proc. Roy. Soc. B 136, 633.

Appendix 1

The sand used for growing the timothy grass was obtained from Levenseat quarry, W. Lothian. Before use it was acid-washed (2% W/V HCl) and then washed acid-free with tap-water before drying at room temperature.

A chemical analysis of the washed sand gave the following results.

CaO	0.01%
Organic matter	nil

A mechanical analysis gave the results

Coarse sand	95.6%
Fine sand	4.4%
Silt	nil

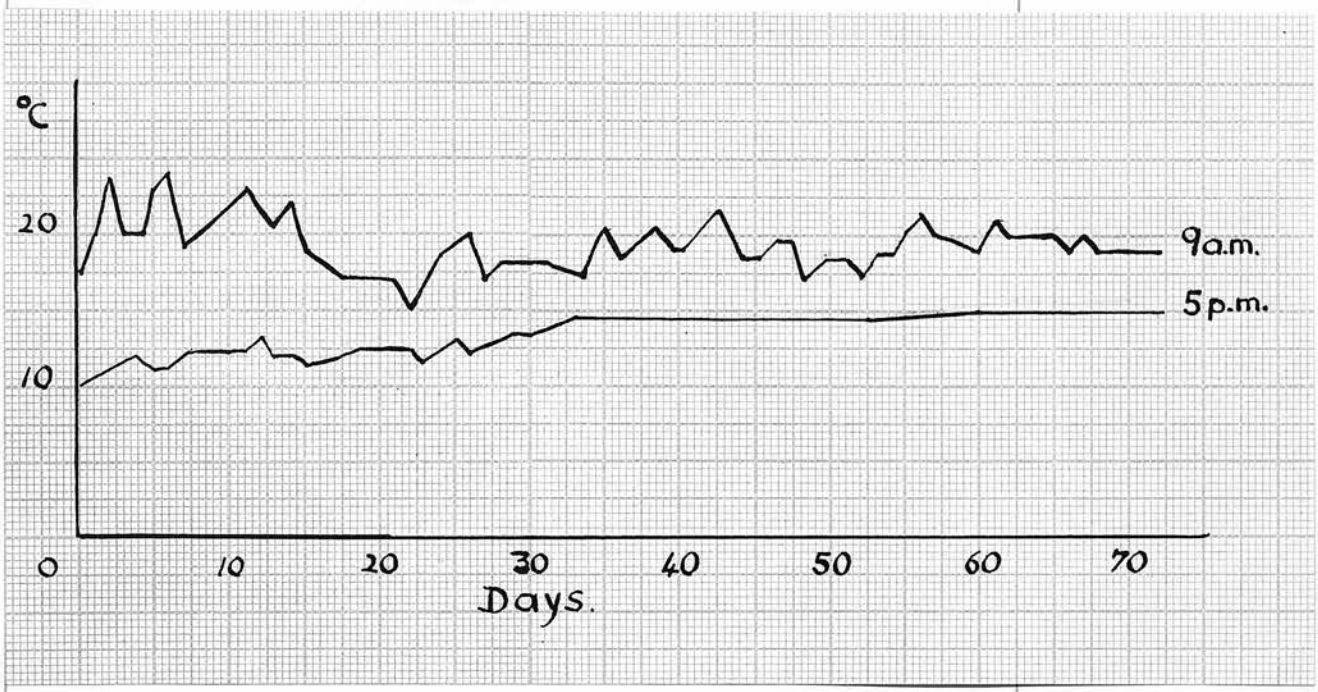
Appendix 2

The nutrient solution employed was of the following composition.

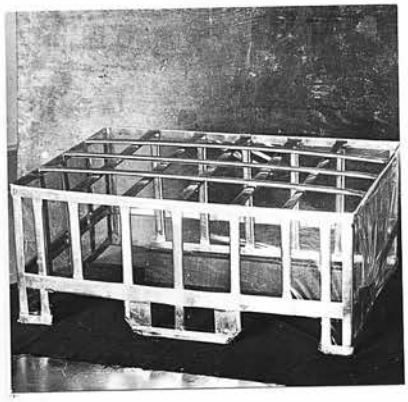
$Ca(NO_3)_2.4H_2O$	0.1N	22 ml.
$Mg(NO_3)_2.6H_2O$	0.1N	33 ml.
$NaNO_3$	1.0N	27.5ml.
K_3PO_4	0.1N	66 ml.
Tap water	-	1850 ml.

The pH was about 7 units and the conductivity at 14^{o} was 1700 $ohms^{-1}$.

Appendix 3

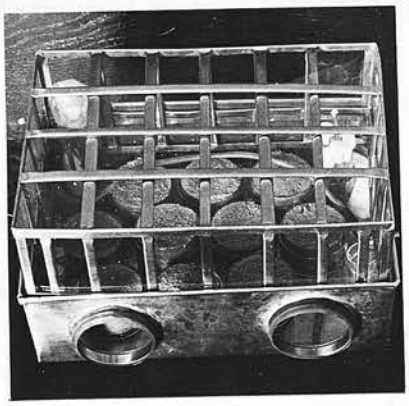


The graphs show the daily temperatures, at the outer surface of the cellophane covering the growth-chamber in Exp.10, taken just before the lights were switched off (9 a.m.) and just before they were switched on (5 p.m.).



1

2



3

4

Plates 1 - 4. Stages in the assembly of the growth-chamber.

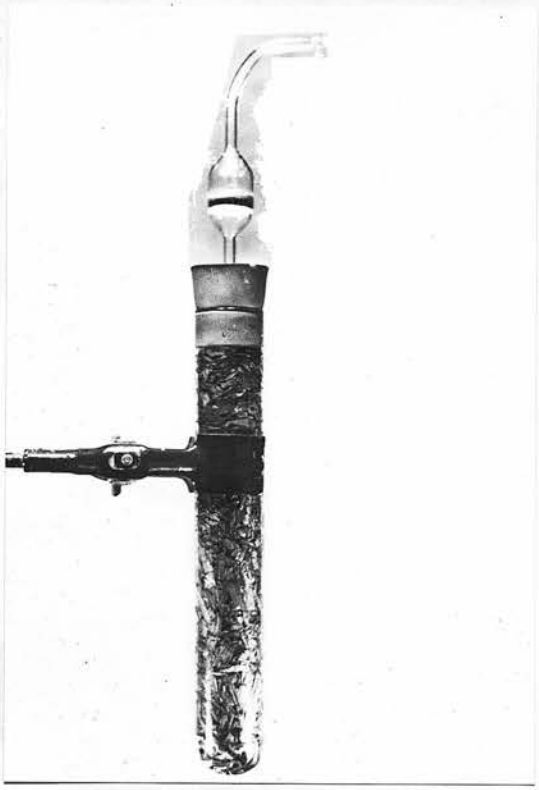


Plate 5. The silo-tube fitted with sintered glass-mercury valve.

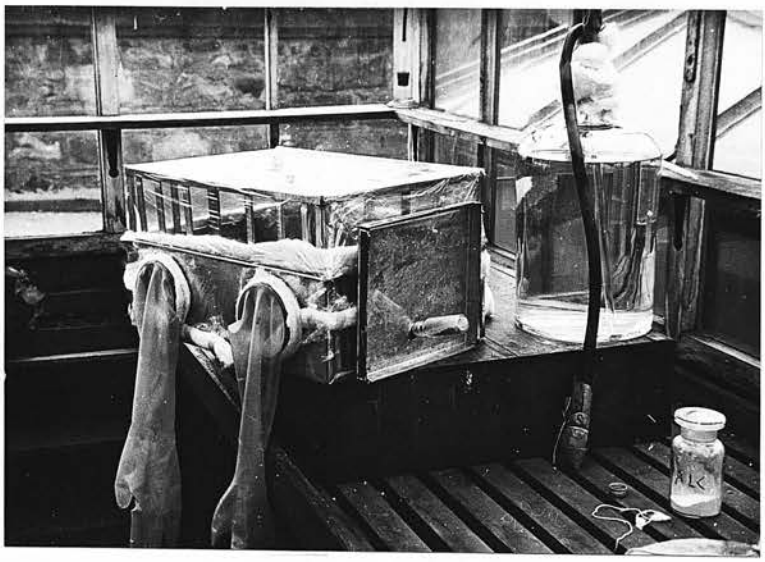


Plate 6. Introduction of the seed into the growth-chamber.

Plate 7. Sowing the seed.

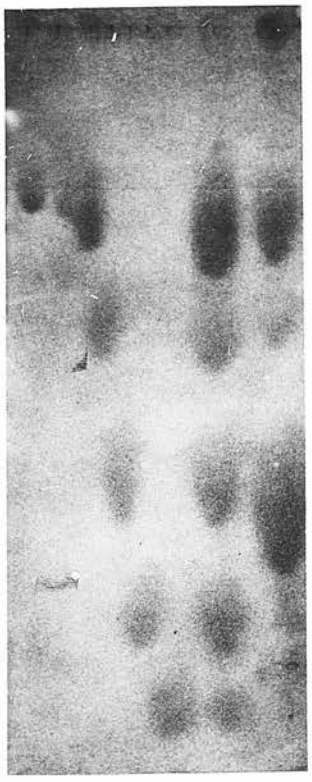


Plate 8. Separation of carboxylic acids on the paper chromatogram by the method of Hiscox & Berridge (1950); left to right: formic, lactic, acetic, propionic, butyric, valeric, caproic acids, mixture of these acids, and a silage extract.

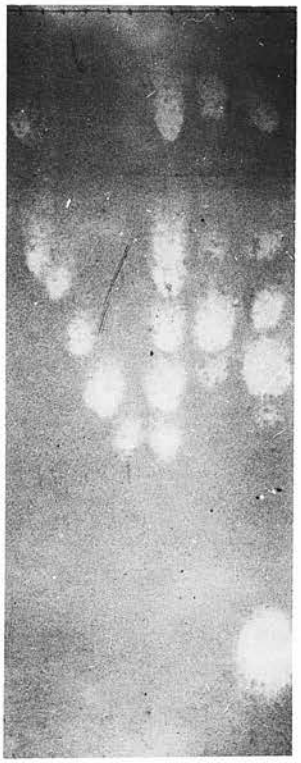


Plate 9. Separation of non-volatile carboxylic acids on the paper chromatogram by the method of Brown (1951); left to right: citric, oxalic, tartaric, malic, succinic, fumaric acids, their mixture, grass extract, and extract of 2-day-old grass silage.

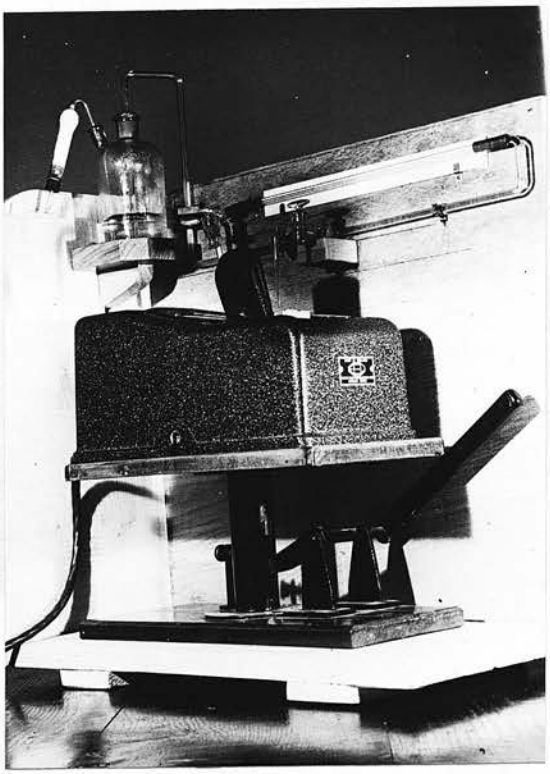


Plate 10. Apparatus used for titrating micro-quantities of lactic acid.

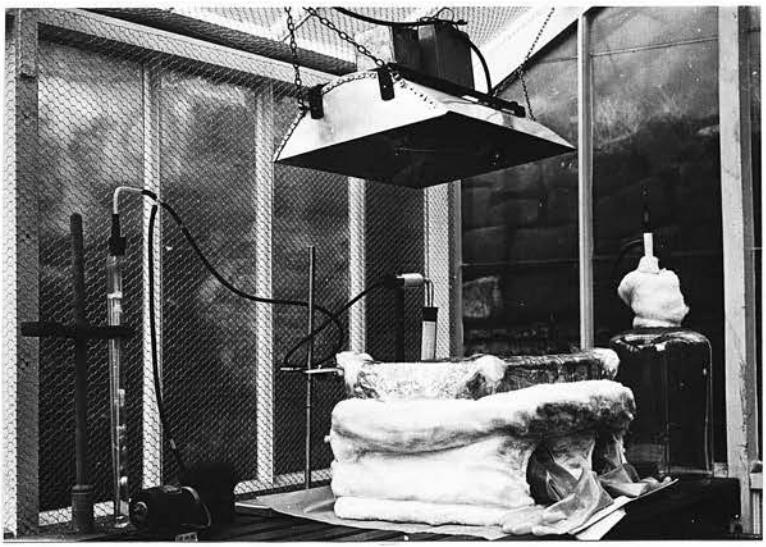
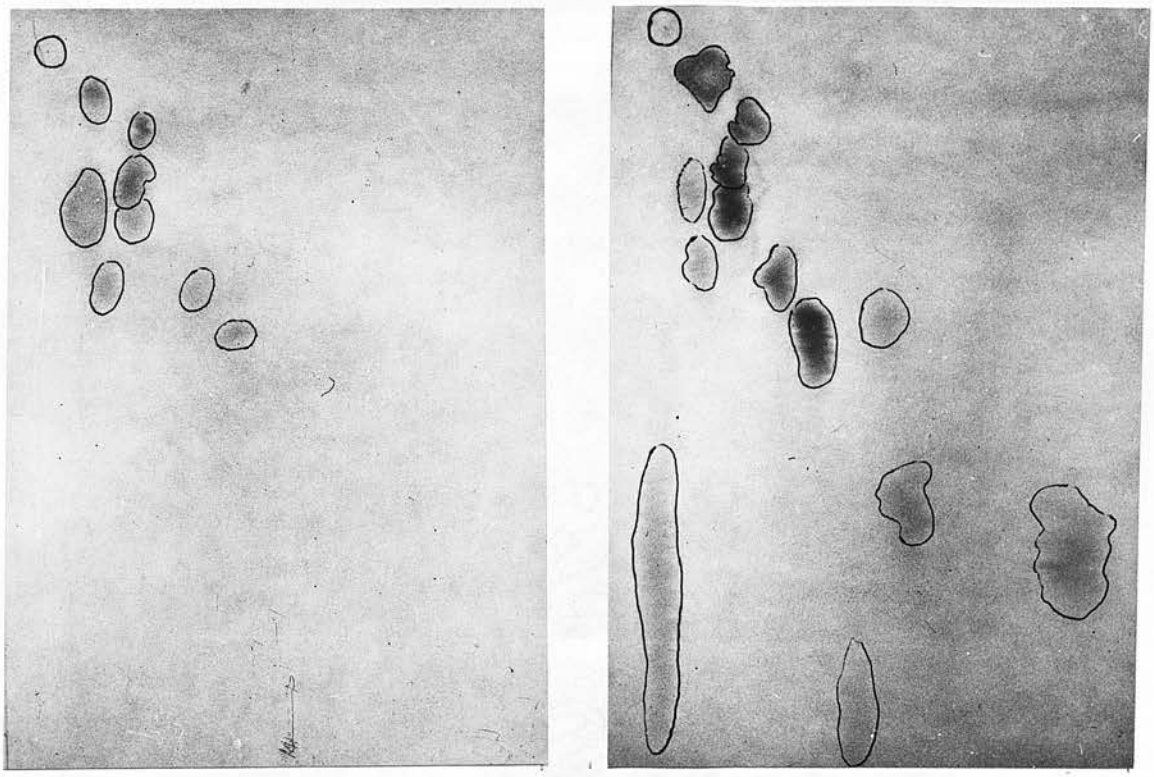


Plate 11. Apparatus used in Exp. 9 in which daylight was supplemented with light from a high-pressure mercury vapour lamp.

Plate 12 has been withdrawn.

Plate 13. Sand cultures of timothy grown under artifical illumination outside (1) and inside (2) of the growth-chamber.



(a) (b)

Plate 14. Paper chromatograms of the amino-acids present in the grass (a) and silage (b) extracts of Exp. 8.

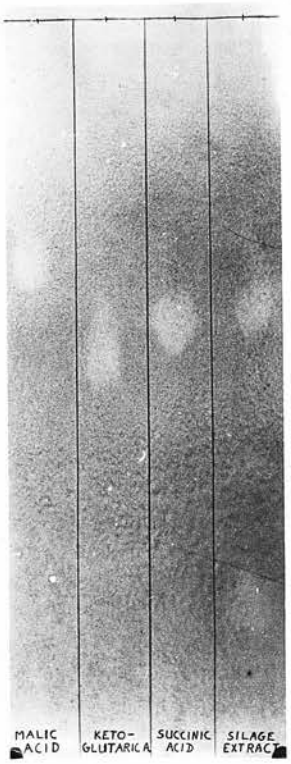


Plate 15. Showing the presence of succinic acid in 10-day-old grass silage.